Two Alternative Egress Pathways for α-Herpesvirinae: The Significance of Glycoprotein K and G to Envelopment

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Summary

Herpesviruses are enveloped, double-stranded DNA viruses. The genome is packed into a capsid which is tegumented before being enclosed by a lipid membrane. Assembly and packaging of capsids occur in the nucleus. Before virus particles are released from the cells, capsids get their tegument and envelope during budding at a cellular membrane compartment. These envelopes bear besides lipids from the host also viral encoded glycoproteins. How herpesviruses get out of a cell and from which cellular compartment they get their envelope has been controversially discussed for years. To address these questions, we used both bovine herpesvirus 1 (BoHV-1) and herpes simplex virus 1 (HSV-1).

In the first part of my thesis we focus on how capsids leave the nucleus. For this purpose we used BoHV-1. In general, capsids are assumed to bud at the inner nuclear membrane. This hypothesis was confirmed by studying infected cells using electron microscopy. The discovery of a continuum from the perinuclear space to the Golgi complex could suggest that capsids, after budding at the inner nuclear membrane, could be intraluminally transported from the perinuclear space to the Golgi cisternae. Finally, virions within Golgi cisternae were packaged into transport vacuoles and were exocytosed. The model of an intraluminal pathway is also supported by the finding of virus particles within the endoplasmic reticulum and Golgi cisternae. However, the detection of naked capsids in the cytoplasm suggests that a pathway must exist to reach this compartment. With high resolution microscopy we found enlarged nuclear pores in cells infected with BoHV-1 compared to mock infected cells. The projection of nuclear material into the cytoplasm through these enlarged nuclear pores implied that capsids utilise these gaps in the nuclear membrane to directly gain access to the cytoplasm. Capsids in the cytoplasm were found to bud at any cellular membrane. All these observations led to the proposal of a new model, according to which BoHV-1 capsids are released from the nucleus by two different pathways, i.e. by budding into the perinuclear space followed by intraluminal transportation as well as by the transportation through dilated nuclear pores. Both pathways coexist in the same infected cell.

To verify, whether our model is also valid for herpesviruses other than BoHV-1, we studied the egress pathway of HSV-1. Furthermore, since glycoprotein K is the only glycoprotein essential for egress of HSV-1 we extended our investigation towards the question for which

step glycoprotein K is required. We generated a glycoprotein K deficient virus by homologous recombination. To get infectious virus particles, the glycoprotein K deficient mutant was propagated on a complementing cell line expressing glycoprotein K upon virus infection. Examination of cells infected with the deficient mutant by electron microscopy revealed naked capsids in the nucleus as well as in the cytoplasm. Although capsids were often encountered close to cellular membranes, enveloped virus particles were missing both in the perinuclear space and Golgi apparatus in the absence of glycoprotein K. Consequently, glycoprotein K is required for proper envelopment of capsids at the inner nuclear membrane as well as at Golgi membranes. Furthermore, the simultaneous observation of capsids in the cytoplasm and enlarged nuclear pores were consistent with the above mentioned model. Hence, our model initially established for BoHV-1 is also valid for HSV-1.

The third part of my thesis focused on the clarification of the unknown function of glycoprotein G of HSV-1. For this purpose, we generated a recombinant virus without the DNA sequences encoding glycoprotein G. Morphological analyses of the Golgi complex revealed an early and significant reduction of the surface area and volume of the Golgi complex in cells infected with the glycoprotein G deficient mutant. Using confocal microscopy, we showed that Golgi complex disaggregated into numerous vesicles in the absence of glycoprotein G. This early breakdown of the Golgi complex could explain all the further phenotypic characteristics in the absence of glycoprotein G. The intraluminal transportation of recombinant virus was blocked due do disassembled Golgi complex, resulting in an accumulation of virus particles within the perinuclear space and endoplasmic reticulum. Another consequence of the Golgi complex breakdown was a reduced virus spread because of diminished envelopment of capsids at the Golgi membranes.

In essence, herpesvirus egress follows two distinct pathways. One population of assembled capsids bud at the inner nuclear membrane and was intraluminally transported to the Golgi complex, whereas the other population utilized the dilated nuclear pores to leave the nucleus. Glycoprotein K is essential for envelopment using either pathway, whereas glycoprotein G protects the Golgi apparatus to prolong the availability of intraluminal egress pathway.

Zusammenfassung

Herpesviren sind behüllte, doppelsträngige DNA-Viren. Ihr Genom befindet sich in einem Kapsid, das von Tegumentproteinen und von einer Hüllmembran umgeben ist. Nach Bildung von Virusnukleinsäure und –proteinen erfolgt im Kern der Wirtszelle die Zusammensetzung der Kapside. Während einer Knospung an einem intrazellulären Membrankompartiment erwirbt das Virus vor der Ausschleusung aus der lebenden Zelle Tegumentproteine und seine Virusaussenhülle. Diese Membran beinhaltet neben den Lipiden der Wirtszelle auch virale Glykoproteine. Auf welchem Weg die Herpesviren die Wirtszelle verlassen und an welchem Kompartiment sie ihre Hülle aneignen, wird seit Jahren in der Literatur kontrovers diskutiert. Diese Fragestellung haben wir mit Hilfe des Bovinen-Herpesvirus Typ 1 (BoHV-1) und Herpes Simplex Virus 1 (HSV-1) versucht zu beantworten.

Der erste Teil meiner Arbeit konzentriert sich auf die Frage, wie die Viruskapside den Kern der Wirtszelle verlassen. Für diesen Zweck verwendeten wir BoHV-1. Allgemein wird angenommen, dass die Kapside an der inneren Kernmembran knospen können. Mittels elektronenmikroskopischen Bildern von infizierten Zellen konnten wir diese Hypothese bestätigen. Die Entdeckung eines Kontinuum vom perinukleären Raum zum Golgi-Komplex lässt uns vermuten, dass die an der inneren Kernmembran knospenden Kapside intraluminal zum Golgi-Komplex transportiert werden, und dort in Transportvesikel verpackt werden. Die Viruspartikeln verlassen nun die Zelle über den Sekretionsweg. Die Beobachtungen von Viruspartikeln im endoplasmatischen Retikulum und in den Golgi Zisternen stützen das Modell des intraluminalen Virustransportes. Trotzdem lässt das Vorfinden von vielen Kapsiden im Zytoplasma vermuten, dass noch ein alternativer Weg zum luminalen Transport bestehen muss. Durch genaue Untersuchungen der Kernmembran unter Anwendung von hochauflösender Mikroskopie, entdeckten wir in infizierten Zellen gegenüber von nicht infizierten Zellen stark erweiterte Kernporen. Das Austreten von Kernmaterial durch diese Kernmembranlöcher lässt uns spekulieren, dass auch Kapside diese Membranlücken benützen können, um auf diesem Weg ins Zytoplasma zu gelangen. Wir beobachteten, dass Kapside im Zytoplasma an intrazellulären Membrankompartimenten knospen, bevor sie freigesetzt werden. All diese Befunde führten zu der Annahme, dass BoHV-1 mittels zwei verschiedenen Mechanismen die Zelle verlassen kann. Unser Modell für den Zellaustritt beinhaltet den

intraluminalen Transport sowie den Transport durch die erweiterten Kernporen. Beide Wege koexistieren in derselben Zelle.

Um zu testen ob unser Modell auch bei anderen Herpesviren Gültigkeit hat, testeten wir die Hypothese anhand von HSV-1. Das Glykoprotein K ist das einzige Glykoprotein, welches für den Zellaustritt unabdingbar ist. Deshalb untersuchten wir im zweiten Teil meiner Arbeit für welchen Schritt des Zellaustritts von HSV-1 das Glykoprotein K essentiell ist. Um dies herauszufinden, erzeugten wir mittels homologer Rekombination ein modifiziertes Virus, bei welchem die kodierende Sequenz für das Glykoprotein K nicht mehr vorhanden ist. Um infektiöse Viruspartikel zu erhalten, wurde das modifizierte Virus auf Zellen propagiert, welche das Glykoprotein K exprimierten und so das Virus komplementierten, sobald die Zelle infiziert wurde. In den mit dem Deletionsmutanten infizierten Zellen weissten wir mit Hilfe des Elektronenmikroskops unbehüllte Kapside im Kern so wie im Zytoplasma nach. Obwohl sich die Kapside oft in der Nähe von Membranen befanden, konnten wir in der Abwesenheit von Glykoprotein K keine umhüllten Viren finden, weder im perinukleären Raum noch im Golgi-Komplex. Daraus folgerten wir, dass das Glykoprotein K für die korrekte Umhüllung der Kapside essentiell ist. Des Weiteren stimmte das zeitgleiche Vorfinden von Kapsiden im Zytoplasma und von erweiterten Kernporen mit unserem oben erwähnten Modell überein. Folglich gilt das anfänglich für den Zellaustritt von BoHV-1 erstellte Modell auch für HSV-1.

Im dritten Teil meiner Arbeit fokussierten wir uns auf die Aufklärung der bis anhin unbekannte Funktion des Glykoproteins G von HSV-1. Für diesen Zweck wurde ein modifiziertes Virus hergestellt ohne kodierende Sequenz für das Glykoprotein G. Morphologische Analysen des Golgi-Komplexes ergaben, dass in Zellen, die mit dem modifizierten Virus infiziert wurden, sowohl die Oberfläche wie auch das Volumen des Golgi-Komplexes stark verkleinert waren bereits zu einem frühen Zeitpunkt der Infektion. Mittels konfokaler Mikroskopie konnten wir zeigen, dass der Golgi-Komplex in Abwesenheit von Glykoprotein G in Vesikel zerfällt. Die frühe Disassemblierung des Golgi-Komplexes kann auch die weiteren phänotypischen Merkmale erklären, die wir in Abwesenheit von Glykoprotein G beobachten konnten. Der intraluminale Transport der modifizierten Viren war aufgrund des disassemblierten Golgi-Komplexes blockiert, was sich in einer Anhäufung von Viren im perinukleären Raum so wie im endoplasmatischen Retikulum widerspiegelte. Eine weitere Folge des frühen Golgi-Komplex Zerfalls war eine geringere Exozytoserate der modifizierten Viren aufgrund einer verminderten Kapsidumhüllung am Golgi-Komplex.

Zusammenfassend lässt sich sagen, dass Herpesviren die Wirtszelle auf zwei verschiedenen Wegen verlassen kann. Eine Population der Herpesviren wird nachdem die Kapside an der inneren Kernmembran umhüllt wurden, intraluminal in den Golgi-Komplex transportiert, die andere Population nützt die entstandenen erweiterten Kernporen um den Kern zu verlassen. Bei beiden Transportwegen ist Glykoprotein K notwendig für die Behüllung, wohingegen Glykoprotein G die Golgi-Komplex Struktur bewahrt und damit den intraluminalen Transport gewährleistet.

Chapter 1

General Introduction

Herpesviridae

Herpesviridae is the name of a family of enveloped, double-stranded DNA viruses (Becker et al., 1968). All herpesviruses are morphologically similar (Fig. 1). The diameter of the entire virion is 170-200 nm (Grunewald et al., 2003). Herpesviruses consist of four elements: an envelope, a tegument, a capsid, and a core (Wildy et al., 1960). The virion is enclosed by a lipoprotein envelope bearing surface projections. The tegument is an amorphous substance composed of viral proteins. The icosahedral capsid consists of 12 pentameric and 150 hexameric capsomers. It has a diameter of 100-110 nm (Newcomb et al., 2003). The DNA, wound in a toroidal shape, makes up the core (Furlong et al., 1972). Characteristically, the herpesvirus genome is not segmented and contains repeated terminal and/or internal sequences (Wadsworth et al., 1975).

More than 200 herpesvirus species have been isolated from men and a wide range of animals, including mammals, birds, reptiles, amphibians and fish (Davison et al., 2005; McGeoch et al., 2000; Quackenbush et al., 1998; Waltzek et al., 2005). Many herpesviruses cause disease in their primary host, and have the ability to remain latent within neuronal, lymphoid or epithelial cells, often persisting for lifetime. The ability to enter latency is an interesting but poorly understood aspect of the herpesvirus life cycle. In the latent state no infectious progeny are produced and only limited transcription of viral gene occurs (Krause et al., 1988; Stevens et al., 1987). The latent virus genome exists in an extrachromosomal form thought to be episomal (Mellerick and Fraser, 1987; Rock and Fraser, 1985). A variety of stimuli such as mental stress, UV reactivation, or menstruation, can reactivate latent viruses leading to a new cycle of productive infection (Hill et al., 1990; Wagner and Bloom, 1997). In the lytic phase, herpesvirus life cycle revolves around: getting into a host cell, replicating there, and getting out again. Acute infection lasts until it is aborted by the host immune system.

Virus transmission commonly occurs by direct contact of mucosal surfaces. Some herpesviruses can be transmitted via body fluids (Burgos et al., 2006; Burgos et al., 2005). Herpesvirus infection begins with attachment to and penetration of a host cell. The viral genome is transported into the nucleus, where replication of the genome takes place (Quinlan et al., 1984). During lytic infection, synthesis of viral gene products is tightly regulated in a sequentially ordered cascade (Honess and Roizman, 1974). Herpesvirus genes, like the genes of their eukaryotic hosts, are not arranged in operons and have individual promoters in most cases (Roizman, 2001). Moreover, very few herpesvirus mRNAs are spliced (Schroder et al.,

1989). The genes are characterized as either essential or dispensable for growth in cell culture. Essential gene products are required for viral entry, transcription regulation, and virion construction. Dispensable gene products for the most part function to enhance the cellular environment for virus production, to defence the virus from the host immune system and to promote cell to cell spread. The large numbers of dispensable genes are in reality required for a productive *in vivo* infection. It is only in the restricted environment of laboratory cell cultures that they are dispensable (Roizman, 1996).

Members of the family of herpesviridae vary greatly with respect to tissue tropism, pathogenicity, and behaviour under culture conditions in the laboratory. The family has been classified into three subfamilies: Alpha-, Beta-, and Gammaherpesvirinae (Roizman and Baines, 1991; Roizman et al., 1981). The Alphaherpesvirinae, which are neurotropic, have a rapid replication cycle. Susceptible cultured cells are quickly destroyed by the infection and propagation of Alphaherpesvirinae. Their host range varies from very wide to narrow *in vivo* as *in vitro*. Betaherpesvirinae are lymphotropic with a long reproductive cycle and restricted host range. They are mostly restricted to the species or genus to which the host belongs. Infected cells frequently become transformed and enlarged (cytomegalia) both *in vivo* and *in vitro*. Gammaherpesvirinae are also lymphotropic and specific for either T- or B-lymphocytes.

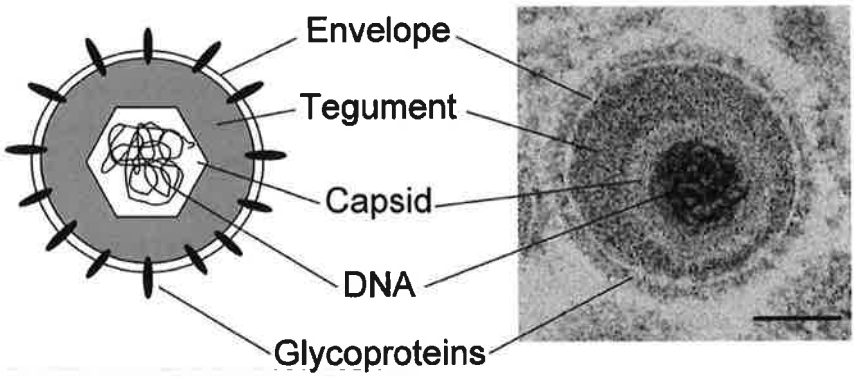

Fig. 1. **Herpesvirus structure.** The virion comprises four features: the viral double-stranded DNA, packaged as a tightly wrapped spool within an icosahedral capsid shell, the tegument consisting of numerous viral proteins, and a lipid bilayer envelope containing virally encoded glycoproteins. The structural components of the virion can be seen in an electron microscopic photograph (right; bar, 100 nm).

Herpes simplex virus type 1 (HSV-1)

HSV-1 is considered to be the archetype of the Alphaherpesvirinae subfamily. HSV-1 infections are among the most common infections worldwide infecting 60% to 90% of the adult world population and cause recurrent infections throughout life (Motani et al., 2006; Whitley and Roizman, 2001). Most primary infections with HSV-1 are asymptomatic. However, in some cases HSV-1 can recur spontaneously in the eye, causing severe lesions that may lead to blindness (Streilein et al., 1997). In very rare cases HSV-1 can spread to the brain, causing herpes encephalitis, a dangerous infection that may lead to death (Skoldenberg et al., 2006; Tyler, 2004). The sensory neurons of the nervous system are the main target cells for HSV-1 infection, in the sense that in the natural cycle of HSV-1 they represent the final cellular destination for the primary infection and the site for establishment of latency (Stevens et al., 1987).

The HSV-1 genome is a 153 kb linear duplex which encodes at least 80 genes. The genome consists of two unique regions, unique long (U_L) comprising 82% and the unique short (U_S) comprising 18%. Both regions are flanked by repeated sequences (Wadsworth et al., 1975). The HSV-1 genome actually exists as an equimolar mixture of four isomeric forms generated by inversion of the U_L and U_S sequences relative to each other (Delius and Clements, 1976; Hayward et al., 1975).

Bovine herpesvirus type 1 (BoHV-1)

BoHV-1 is also a member of the Alphaherpesvirinae subfamily and shares some biological properties with HSV-1 such as the ability to infect neurons and establish latency (Jones, 1998). BoHV-1 is considered the most important etiologic agent of bovine respiratory diseases that cause major economic losses in cattle industries (Ackermann and Engels, 2006; Bowland and Shewen, 2000). Primary infection is accompanied by various clinical manifestations such as infectious bovine rhinotracheitis, abortion, infectious pustular vulvovaginitis, and systemic infection in neonates (Chow et al., 1964; Engels and Ackermann, 1996; Owen et al., 1964; Schwyzer and Ackermann, 1996; Tikoo et al., 1995). When animals survive, a lifelong latent infection is established in ganglionic neurons of the peripheral nervous system. Reactivation from latency and spread to other susceptible animals occur after natural or corticosteroid-induced stress (Pastoret et al., 1986; Rock et al., 1992; Sheffy and

Davies, 1972). BoHV-1 has also the ability to infect and provoke clinical symptoms in heterologous host like goats or sheep (Elazhary et al., 1984).

The arrangement of BoHV-1 genome is similar to the HSV-1 genome (Fig. 2) except that only the U_S region is bracketed by inverted internal and terminal repeated sequences (Roizman, 2001). The genome of BoHV-1 is 135 kb long and exhibits a GC ratio of 72.4% (Plummer et al., 1969). BoHV-1 genome encodes at least 69 genes the majority of the BoHV-1 genes are homologous to genes found in HSV-1 and other alphaherpesviruses (Schwyzer, 1995; Schwyzer et al., 1996).

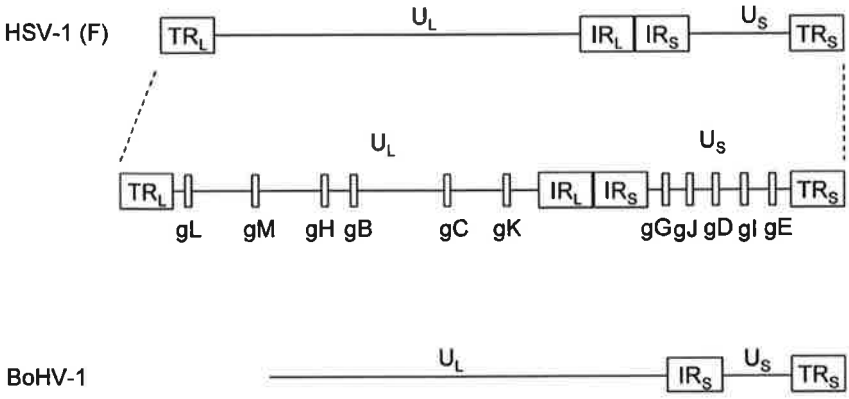

Fig. 2. **The herpesvirus genome.** The Herpesvirus genome is organized into two components, unique long (U_L) and unique short (U_S). The unique regions of HSV-1 are flanked by inverted repeat regions (internal repeat, IR; terminal repeat, TR) and are linked to generate a total genome size of approximately 153 kbp. The localisation of the 11 genes encoding HSV-1 glycoproteins is indicated below.
In the genome of BoHV-1 only the U_S region is flanked by inverted repeats. The total size of BoHV-1 genome is approximately 135 kpb.

Herpesvirus life cycle

The lytic cycle can be divided into three parts, namely the cell entry the replication and the egress.

Cell entry

Since herpesviruses are obligate intracellular parasites, they have to enter cells for the initiation of infection. Entry into a cell involves the function of a number of viral envelope proteins, including at least glycoprotein B (gB), gC, gD, and the gH/gL heterodimer. When a herpesvirus particle lands on a cell, gC docks to heparin sulphate moieties on the host cells (Herold et al., 1991). However, this interaction appears to be more of a facilitator for infection rather than a strict requirement. Neither the absence of gC on the viral envelope nor the lack of heparin sulphate on cells can prevent virus infection (Shieh et al., 1992; WuDunn and Spear, 1989). Virion attachment is stabilized by the binding of gD to cellular specific receptors. To date, three classes of cell receptors for HSV-1 gD have been identified on mammalian cells. They include herpesvirus entry mediator, a member of the tumor necrosis factor receptor family (Geraghty et al., 1998; Montgomery et al., 1996); nectin-1 and nectin-2, two members of the immunoglobulin superfamily (Cocchi et al., 1998); and specific sites in heparin sulphate generated by certain isoforms of 3-O-sulfotransferases (Shukla et al., 1999). The following steps after gD binding to a cellular receptor, which leads to viral penetration, is less well understood. Cell entry is assumed to occur by fusion of the virion envelope with the cell membrane releasing both tegument and capsid into the cytoplasmic matrix (Turner et al., 1998; Wittels and Spear, 1991). Alternatively, endocytosis of HSV-1 is suggested for some cell types (Nicola et al., 2003; Nicola and Straus, 2004). However, Wild et al. examining more than 200 virions entering cells found neither indications for fusion between viral envelope and plasma membrane nor indications for endocytosis. The group claimed that intact virions enter the cytoplasm through a hole in the plasma membrane initiated by the virus interaction to the plasma membrane (Wild et al., 2007). Once virus particles have entered the cytosol of a cell, they must be transported towards the nucleus by using dynein motor complex associated with microtubules (Dohner et al., 2002). Release of viral DNA into the nucleoplasm is assumed to occur at the nuclear pore (Ojala et al., 2000; Sodeik et al., 1997). While the capsid remains in the cytoplasm, some tegument proteins such as VP16 enter the nucleus together with the viral genome (LaBoissiere and O'Hare, 2000).

Virus replication

The viral DNA is thought to circularize upon entry into the nucleus and in the lytic cycle, transcription of the viral genes begins (Su et al., 2006). Three classes of viral gene expression kinetics have been identified: immediate-early, early, and late. While host RNA polymerase II

is responsible for synthesis of all viral mRNAs (Alwine et al., 1974) viral proteins are necessary for the initiation and enhancement of transcription of certain genes (Batterson and Roizman, 1983). A complex of VP16, which is supplied as tegument protein into infected cells, and cellular proteins induces the immediate-early genes which are transcribed immediately following viral entry in the absence of de novo protein synthesis (Campbell et al., 1984). Activation of viral genes is accompanied by a corresponding decrease in cellular gene expression, a phenomenon known as host shutoff (Feng et al., 2001; Strand et al., 2004). Immediate-early proteins appear to be the key players in the inhibition of host transcription (Kemp and Latchman, 1988; Spencer et al., 1997). Furthermore, immediate-early proteins serve as transactivators and switch on the expression of the early genes which are made before DNA replication. The proteins made by the expression of the early genes include enzymes involved in DNA metabolism and replication. The late genes are expressed after DNA replication. They are encoded to make structural proteins as well as elements of the tegument (Honess and Roizman, 1974).

Both immediate-early and early proteins are required for genome replication (Thiry et al., 2005). The circular DNA molecule proved the template for HSV-1 replication together with a number of viral enzymes and cellular proteins. Replication starts from sequences known as origins of replication. The pattern of replication is complex, beginning with theta bidirectional replication and then switch to the rolling circle mode of replication to produce high molecular weight DNA concatemers (Boehmer and Lehman, 1997; Schynts et al., 2003).

Virus assembly

Herpesvirus assembly is a multistep process occurring at late times in infection (Newcomb et al., 1996). The first stages of virus assembly take place in the nucleus of infected cells. The viral capsid requires synthesis of numerous late proteins, which first assembled as capsid precursors, called procapsids. Procapsids do not contain DNA and consists of an inner scaffold, not found in the mature virion, and an outer capsid shell that persists as a component of all mature capsid types and infectious virions (McClelland et al., 2002). Viral packaging proteins bind to specific sites in the newly synthesised concatemeric DNA and subsequently cleave the duplex DNA to generate one mature genome end. The packaging protein with the attached concatemeric DNA then binds to the portal complex of a procapsid and translocates the viral genome into the procapsid. During DNA packaging the scaffold is cleaved by the viral protease and exits the procapsid or is degraded (Deiss et al., 1986; Ladin et al., 1982; Varmuza and Smiley, 1985). DNA-filled capsids then leave the nucleus and acquire tegument

and envelope before exiting the cell. However, it is a matter of debate how alphaherpesvirus mature capsids leave the nucleus and how these particles are transported to the cell surface. Two models have been proposed for HSV-1 egress; the reenvelopment model (Skepper et al., 2001) and the vesicle formation model (Campadelli-Fiume et al., 1991). Both models propose that capsids bud at the inner nuclear membrane and that the Golgi complex is involved in maturation of the virions, and that finally mature virions leave the cell by exocytosis via Golgi-derived vesicles.

The reenvelopment model

In this model, the primary envelope is acquired by budding of DNA-containing capsids through the inner nuclear membrane. Some tegument proteins and a glycoprotein studded envelope are part of these virions in the perinuclear space, named primary virions (Baines et al., 1995; Baines et al., 2007; Fuchs et al., 2002a; Reynolds et al., 2002). Primary virions contain an electron lucent tegument and a dense envelope (Granzow et al., 2001). In order to gain access to the cytoplasm, the virions fuse with their primary envelope to the outer nuclear membrane, thereby releasing naked capsids into the cytoplasm (Smith, 1980; Stackpole, 1969). Once translocated into the cytoplasm, naked capsids definitively acquire their mature tegument and secondary envelope by budding into a presumably *trans*-Golgi compartment (Harley et al., 2001; Turcotte et al., 2005). Virions within vesicles derived from *trans*-Golgi network are then transported to the plasma membrane, where the membrane of vesicles and the plasma membrane fuse and mature virions are released into the extracellular space (Fig. 3).

Some evidence for the reenvelopment theory comes from the failure to detect the same composition of the tegument between primary virions within the perinuclear space and mature virions. Some tegument proteins which are present in virions in the perinuclear space have not been detectable on extracellular virions and vice versa (Fuchs et al., 2002b; Miranda-Saksena et al., 2002). Differences in the phospholipids composition between mature HSV-1 virions and the nuclear membrane (van Genderen et al., 1994) as well as the different appearance (Granzow et al., 2001) of virus morphology are used as further evidences. However, the inability to show fusion events of the viral envelope with the outer nuclear membrane is one remaining difficulty to generally accept the reenvelopment as unique model for herpesvirus egress.

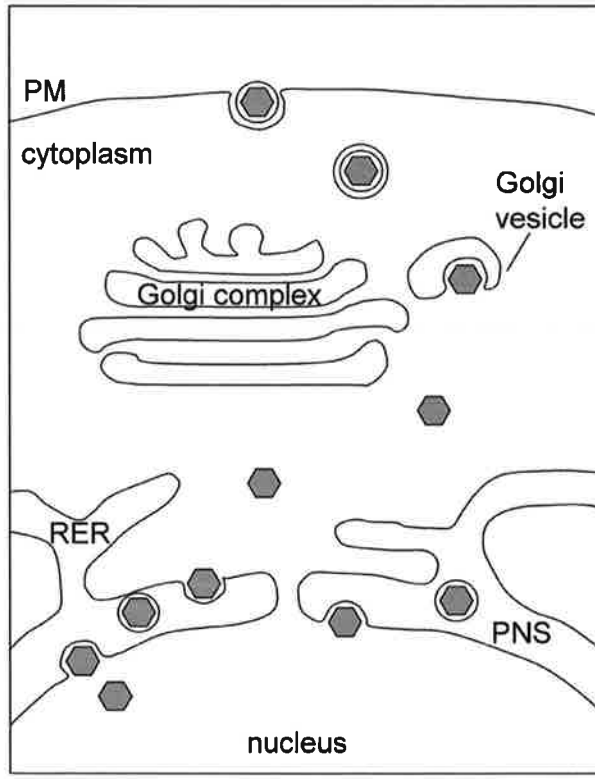

Fig. 3. Diagram of the proposed reenvelopment pathway of herpesvirus egress. Nucleocapsids bud into the perinuclear space (PNS). The primary envelope fuses with the outer nuclear membrane, releasing capsid into the cytoplasm. Cytoplasmic capsids acquire their mature tegument and secondary envelope by budding into a *trans*-Golgi compartment leading to enveloped virions within transport vesicles. Mature virions were released from the cell by exocytosis. PM, plasma membrane; RER, rough endoplasmic reticulum.

The vesicle formation model

This theory assumes that following capsids budding at the inner nuclear membrane, virions leave the perinuclear space via vesicles formed at the outer nuclear membrane or at the rough endoplasmic reticulum (Campadelli-Fiume et al., 1991). The envelope gained at the inner nuclear membrane is retained on virions. Vesicles, which contain the enveloped virions, are transported towards the Golgi complex. Thereafter, the vesicular membranes fuse with Golgi

membranes to release virus particles into Golgi apparatus (Poliquin et al., 1985). Immature glycoproteins on the viral envelope acquired by budding at the inner nuclear membrane are modified within the Golgi apparatus providing the machinery for glycoprotein maturation (Di Lazzaro et al., 1995; Torrisi et al., 1992). Finally, virus particles leave the cell by exocytosis from Golgi- derived vesicles (Fig. 4).

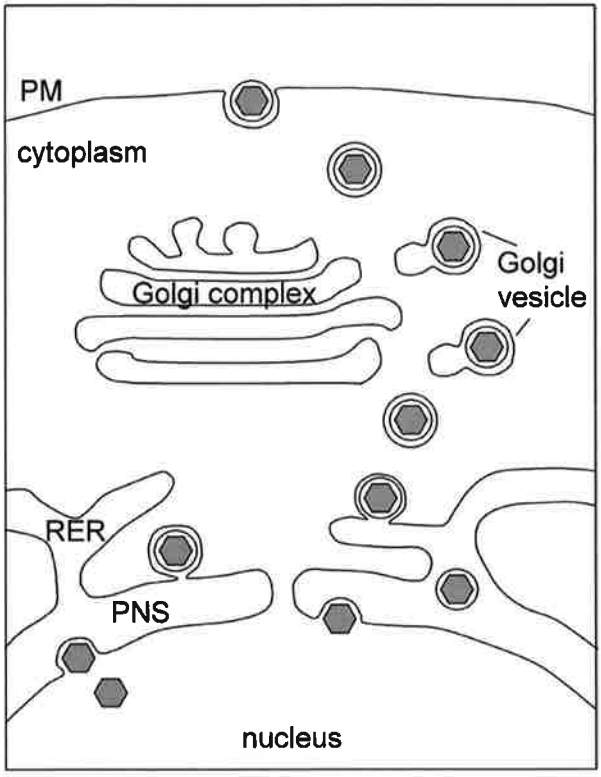

Fig. 4. Diagram of the proposed vesicle formation pathway of herpesvirus egress. The viral envelope is acquired by budding of nucleocapsids through the inner nuclear membrane. The perinuclear virions exit the perinuclear space (PNS) by vesicle formation at the outer nuclear membrane or at the rough endoplasmic reticulum (RER). Vesicles containing virions pass the Golgi complex for final maturation of virions. Finally, virions are released into the extracellular space by exocytosis. PM, plasma membrane.

According to the vesicle formation route of egress, virions always remain in luminal space and virions maintain the envelope acquired at the inner nuclear membrane. Furthermore, unenveloped capsids in the cytoplasm are considered as 'dead-ends', formed by uncontrolled fusion of the viral envelope with the membrane of the surrounding vesicle (Campadelli-Fiume et al., 1991).

The main argument for this model is the detection of oligosaccharides at intermediate steps of maturation on virus particles within intracellular transport vesicles (Di Lazzaro et al., 1995). Furthermore, extracellular virus particles carrying *cis*- and *medium*- Golgi markers support the model of a stepwise viral maturation along the exocytic pathway (Miranda-Saksena et al., 2002).

Glycoproteins

The genome of HSV-1 includes eleven genes coding for glycoproteins localized on the outer membrane of the virion. The viral envelope contains 600-750 glycoprotein spikes and their distribution in the envelope suggests functional clustering (Grunewald et al., 2003). Glycoproteins were named gB, gC, gD, gE, gG, gI, gJ, gK, gL and gM (Fig. 2) and are processed by signal sequence cleavage. *N*- as well as *O*-linked oligosaccharides are attached to these envelope proteins and they may form homo- or heterodimers (Cai et al., 1988; Claesson-Welsh and Spear, 1986; Hutchinson et al., 1992). The majority of HSV-1 glycoproteins are type I membrane proteins. Exceptions include gL, which is soluble (Hutchinson et al., 1992), gB, gK and gM which traverse the membrane several times (McGeoch et al., 1988; Mo and Holland, 1997; Pellett et al., 1985). Many of the glycoproteins serve multiple functions at different steps in the viral replication cycle. They play an important role in the interactions between viruses and their host-cells. Furthermore, because of their localization in the virion envelope and on the surface of infected cells the glycoproteins are important targets for the host immune response (de Boer et al., 1994; Mitchell and Stevens, 1996).

Six of the glycoproteins seem to be dispensable for replication in cell culture (gC, gE, gG, gI, gJ, and gM). However, important functions are attributed to them *in vivo* for example interfering with the immune response. For example gI and gE are suggested to form a Fc receptor (Johnson and Feenstra, 1987; Lubinski et al., 1998). In addition, the gE/gI complex and gM interact with the receptors at cell junctions in order to facilitate cell-to-cell spread of the virus (Crump et al., 2004; Farnsworth and Johnson, 2006). gG homologues of several

alphaherpesviruses can bind a broad range of chemokines with high affinity and thus inhibit their biological activity (Bryant et al., 2003). In contrast, gG of HSV-1 lacks chemokine binding activity and the function has been resistant to detailed characterisation. gJ performs the task of blocking the apoptotic cascade thereby preventing cell death (Zhou et al., 2000). gC has been shown to bind complement factor C3b (Eisenberg et al., 1987). Furthermore, gC mediates the initial attachment of the virus to the cell surface (Herold et al., 1991).

gB is the most conserved glycoprotein among herpesviruses and plays an essential role in virus entry into target cells together with gD and the gH/gL heterodimer complex (Browne et al., 2001; Spear, 2004; Subramanian and Geraghty, 2007). gD interacts with cellular receptors while gB and gH/gL are required for penetration of the host membrane (Crump et al., 2004; Farnsworth and Johnson, 2006). Additionally, gL may interact with specific cellular chemokine receptors during the invasion of HSV-1 (Wyrwicz and Rychlewski, 2006). It is interesting to note that none of the HSV-1 glycoproteins including gB, gD, gH and gL, which are required for virion infectivity has yet been shown to be necessary for virion assembly or envelopment. However, gK plays an essential role in the virion transport to the cell surface (Foster and Kousoulas, 1999; Melancon et al., 2005).

Despite of the remarkable progress in characterising of the glycoproteins in past years there is still lack of detailed information. Many important questions still remain unresolved.

Aim of the present work

The egress of herpesviruses has been controversially discussed since the first reports in the 1970ties. Two pathways have been suggested for herpesvirus egress; the reenvelopment pathway and the vesicle formation pathway. Options differ about the route by which virions leave the perinuclear cisternae. It might seem simple to distinguish between these two alternatives, but this is not the case. Both models have their evidences as well as their inconsistencies.

The aim of this thesis was to shed light on the egress pathway. We extensively analysed the nuclear surface of infected cells by high resolution microscopy. The finding of dilated nuclear pores in infected cells as well as fully enveloped virions in the perinuclear space and rough endoplasmic reticulum lead us to the hypothesis that herpesvirus envelopment follows two diverse pathways. Our egress model was named dual-pathway.

Virion morphogenesis and egress as well as glycoprotein transport are highly dependent on preservation and optimisation of the Golgi apparatus. Given the fact that herpesviruses

possess multiple strategies to modify the cellular milieu to their advantage, it can be argued that herpesviruses must encode mechanisms responsible for the optimisation of intracellular organelles such as the Golgi apparatus as well as cellular membrane trafficking systems to ensure efficient virion maturation and egress. Such virally encoded strategies must involve viral glycoproteins, which may not only regulate specific steps in virion maturation, but may also have important roles in the preservation and enhancement of intracellular organelles and membrane-trafficking systems.

An extended literature search of factors responsible for protective function in the egress pathway resulted in the hypothesis that glycoprotein gK as well as gG may execute important roles in efficient virion assembly. Therefore, gK and gG were exploited to examine the route of viral egress. Recombinant viruses with a gK deletion or a gG deletion were constructed and analysed.

In the absence of gK, no infectious virus particles were formed due to a defective acquisition of the viral envelope. Despite the lack of successful budding at the inner nuclear membrane, naked capsids were found throughout the cytoplasm. These observations indicate that capsids gain access to the cytoplasm other than through fusion events at the outer nuclear membrane. Indeed, large gaps in the nuclear membrane were found suggesting that these gaps represents impaired nuclear pores and that capsids enter the cytoplasm through these impaired nuclear pores.

In the absence of gG, a significant reduction of volume and surface area of the Golgi complex could be observed early in infection. As a consequence, intracellular transportation of virus particles was impaired. Thus, gG is necessary for the integrity of the Golgi complex during HSV-1 infection and fosters its transportation capacity.

In summary, the phenomena observed in the absence of gK or gG are most consistent with the proposed dual-pathway model.

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Chapter 2

Impairment of Nuclear Pores in Bovine Herpesvirus 1-Infected MDBK Cells

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Abstract

Herpesvirus capsids originating in the nucleus overcome the nucleocytoplasmic barrier by budding at the inner nuclear membrane. The fate of the resulting virions is still under debate. The fact that capsids approach Golgi membranes from the cytoplasmic side led to the theory of fusion between viral envelope and outer nuclear membrane, resulting in release of capsids into the cytoplasm. We recently discovered a continuum from the perinuclear space to the Golgi complex implying (i) intracisternal viral transportation from the perinuclear space directly into Golgi cisternae and (ii) the existence of an alternative pathway of capsids from the nucleus to the cytoplasm. Here, we analyzed the nuclear surface by high-resolution microscopy. Confocal microscopy of MDBK cells infected with recombinant bovine herpesvirus 1 expressing green fluorescent protein fused to VP26 (a minor capsid protein) revealed distortions of the nuclear surface in the course of viral multiplication. High-resolution scanning and transmission electron microscopy proved the distortions to be related to enlargement of nuclear pores through which nuclear content including capsids protrudes into the cytoplasm, suggesting that capsids use impaired nuclear pores as gateways to gain access to the cytoplasmic matrix. Close examination of Golgi membranes, rough endoplasmic reticulum, and outer nuclear membrane yielded capsid-membrane interaction of high identity to the budding process at the inner nuclear membrane. These observations signify the ability of capsids to induce budding at any cell membrane, provided the fusion machinery is present and/or budding is not suppressed by viral proteins.

Introduction

Herpesviruses comprise the capsid containing the viral genome, the viral envelope consisting of a lipid bilayer with embedded glycoproteins, and tegument proteins filling the space between capsid and envelope. DNA double strands formed during replication are packed into capsids built of proteins imported from the cytoplasm (Rixon, 1993). Capsids are transported to the nuclear periphery. Their pathway through the nucleocytoplasmic barrier and the acquisition of tegument and envelope are yet not fully understood (Homman-Loudiyi et al., 2003). Capsids bud through the inner nuclear membrane into the perinuclear space, concomitantly acquiring an envelope (Granzow, 2001) and tegument proteins (Wild et al., 2002). It is assumed that the envelope derived from budding at the inner nuclear membrane fuses with the outer nuclear membrane releasing both tegument and capsid into the cytoplasmic matrix. (Browne et al., 1996; Card et al., 1993; Church and Wilson, 1997; Gershon et al., 1994; Gong and Kieff, 1990; Granzow, 2001; Granzow et al., 1997; Klupp et al., 1998; Whealy et al., 1991; Zhu et al., 1995). Capsids are then transported to the *trans*-Golgi network, where they are wrapped by Golgi membranes leading to an enveloped virion within a transport vacuole. Alternatively, it is speculated that virions escape from the perinuclear space via vesicle formation at the outer nuclear membrane (Campadelli-Fiume et al., 1991; Church and Wilson, 1997; Gershon et al., 1973; Granzow et al., 1997; Radsak et al., 1996; Torrisi et al., 1992). These vesicles then pass the Golgi complex for final maturation of virions. Contradictory to both the fusion and vesicle formation theory is the fact that fully enveloped virions were found in the perinuclear space and rough endoplasmic reticulum (RER) (Gilbert et al., 1994; Granzow et al., 1997; Radsak et al., 1996; Roller et al., 2000; Schwartz and Roizman, 1969; Whealy et al., 1991), implying that virions are transported within the RER system. Connectivity between RER and the Golgi complex (O'Donnell et al., 1988; Wang et al., 2001; Wild et al., 2002) makes it very likely that virions are transported from the perinuclear space via the RER into the Golgi complex for packaging into transport vacuoles (Wild et al., 2002). If this theory of intracisternal transport is correct, an alternative pathway for capsids entering the cytoplasmic matrix must exist as has been postulated for herpes simplex virus 1 (Brown et al., 1994) and simian agent 8 (Borchers and Oezel, 1993).

The amount of membranes required for budding is enormous, considering that more than 1,000 capsids may be produced within a single nucleus. If the fusion theory is correct, membrane constituents would have to recycle from the outer to the inner nuclear membrane. If the vesicle formation theory were correct, membranes would have to be supplied for both

the viral envelope deriving from the inner membrane and the vesicle membrane originating at the outer nuclear membrane. If the intracisternal transportation theory were correct, membrane constituents lost by budding at the inner nuclear membrane would have to be replaced. In any case, dramatic membrane translocation would be expected at one or both nuclear membranes while release of capsids via budding is going on.

Cells infected with bovine herpesvirus 1 (BoHV-1) disintegrate in the course of virus multiplication. This most likely involves fast disintegrating processes. The rapidity of cell disintegration suggests dramatic distortions within the nucleus and/or at the nuclear envelope. Such fast events can only be recognized by electron microscopy, provided the temporal resolution of the preparation methods applied is sufficient. In addition, structures must be kept in place during the follow-up preparation or during observation in the electron microscope to visualize disintegrating processes such as the breakdown of membranes. High temporal resolution sufficient to study processes that take place in the millisecond range is achieved by rapid freezing of cellular or subcellular material down to liquid nitrogen temperatures. Loss of material can be prevented by examination of samples in the frozen hydrated state either by cryo-transmission electron microscopy (cryo-TEM), cryoscanning electron microscopy (cryo-SEM), or by transmission electron microscopy (TEM) of specimens dehydrated at low temperatures (LTEM) (Weibull and Christiansson, 1986).

To identify nuclear distortions, we investigated living cells infected with green fluorescent protein (GFP)-labeled BoHV-1 by confocal laser scanning microscopy to get an idea about the time course of virus multiplication in individual cells and the rapidity of their disintegration and correlated the findings by conventional TEM (CTEM). We then reconstructed nuclei from images obtained by confocal microscopy after fixation and DAPI (4',6'-diamidino-2-phenylindole) staining, and employed cryo-field emission SEM (cryo-FESEM) of rapidly frozen, freeze-fractured, and platinum-shadowed cells (Walther et al., 1995). Finally, the nuclear surface and behavior of cell membranes were investigated at high resolution by LTEM. Here, we show that nuclear pores become impaired in the course of BoHV-1 multiplication while nuclear membranes and the plasma membrane seem to remain morphologically intact until the onset of disintegration. Distortion of nuclear pores seems to function as a gateway for capsids to pass the nucleocytoplasmic barrier rather than to play an essential role in cell disintegration.

Material and Methods

Viruses and cells. Madin Darby bovine kidney (MDBK) cells were grown in Dulbecco's modified Eagle's medium (D'MEM: Gibco, Bethesda, MD, USA) supplemented with 10% fetal calf serum (Gibco) at 37°C and 5% CO_2. Wild-type (wt) BoHV-1 (strain Jura) (Metzler et al., 1986) and recombinant BoHV-1 were propagated in MDBK cells as described elsewhere (Fraefel et al., 1994).

Generation of recombinant BoHV-1. BoHV-1 specific sequences flanking the open reading frame of UL35 (encoding for VP26) were amplified by PCR in two separate amplification reactions with primer pairs UL35+ (5'-CGC ATG CAT GGC GTC GTC GAA CCG CGA GTG-3') and UL34- (5'-CCA TCA CGG CGG AGC AGA ACA ACG CC-3') or UL35- (5'-ACG CCA TGC ATG CGC CTG CCG GGA TCG ACC-3') and UL36+ (5'-GCG CGG CAC GGG CAT AAG CGT CCT GG-3'). Both amplification products were cloned and combined in pGEM-3Zf(+) such that a single NsiI site was introduced at the initiation codon of UL35. By site-directed mutagenesis, a single XhoI site was inserted between the putative poly(A) signal of UL35 and UL36, which was subsequently used for the insertion of a tetracycline-resistant gene under the control of a bacterial promoter amplified from pBR322 using the primer pair TET+ (5'-CTT CTC GAG TTC TCA TGT TTG ACA GCT TAT-3') and TET- (5'-GGA CTC GAG AAA AAT CAC TCA GGG TCA ATG-3'). The resulting plasmid was designated p129. The GFP gene was amplified by PCR from pEGFP-N3 (Clontech) using the primer pair GFP+ (5'-CAC CAT GCA TAG CAA GGG CGA GGA GCT GTT C-3') and GFP- (5'-GCC CTG CAG CTT GTA CAG CTC GTC CAT GCC-3'). The ends of the PCR product were digested with NsiI and PstI and ligated into p129, resulting in p131. The linear fragment of p131 comprising the fusion protein gene GFP-UL35 and the gene for tetracycline resistance under the control of a bacterial promoter, flanked by BoHV-1 specific sequences was amplified by PCR primer pair UL36+ and UL34-. The plasmid DNA, which served as template for the PCR amplification, was subsequently digested with DpnI, and DpnI resistant amplification product was used for insertion into electrocompetent and arabinose-induced *Escherichia coli* strain DH10B harboring fl15.003, the wild-type BoHV-1 bacterial artificial chromosome (BAC) (Tobler et al., 2002), and pKD46, which were selected on LB agar plates containing tetracycline. Recombinant BoHV-1 BAC DNA was amplified in *E. coli* and purified.

Recombinant BoHV-1 was reconstituted by introduction of recombinant BoHV-1 BAC DNA into MDBK cells as previously described (Tobler et al., 2002). The genomic integrity of recombinant BoHV-1 expressing the GFP-VP26 fusion protein (rBoHV-1-GFP-VP26) was analyzed by restriction enzyme digestion and PCR analysis. The functionality of the fusion protein gene was proven by fluorescence microscopy.

Infection of cells. MDBK cells were grown for 2 days either in petri dishes with a glass slide bottom (Mattek, Ashland, Mass) for confocal time-lapse microscopy on cover slips for confocal analysis of nuclear alterations, in petriPERM dishes (Sartorius, Göttingen, Germany) for cryo-FESEM, or on 30-µm-thick sapphire disks with a diameter of 3 mm (Bruegger, Minusio, Switzerland) for LTEM, correlative confocal microscopy, and CTEM. Dishes, coverslips, and sapphire disks were covered with 8- to 10-nm-thick carbon obtained by evaporation under high-vacuum conditions to enhance cell growth. Then cells were infected with BoHV-1 at a multiplicity of infection (MOI) of 1, 5, and 10 or with rBoHV-1-GFP-VP26 (MOI, 0.5), and kept at 4°C for 1 h to admit adsorption prior to incubation at 37°C for up to 42 h.

Confocal microscopy. Time-lapse microscopy was performed with a confocal laser scanning microscope (model SP2; Leica, Mannheim, Germany) equipped with an environmental chamber at 37°C and 5% CO_2. For nuclear analyses cells were fixed with 4% paraformaldehyd and stained with DAPI in phosphate-buffered saline (1 µg/ml). Samples were embedded in fluorescence mounting medium (DakoCytomation, Glostrup, Denmark) and analyzed by confocal laser scanning microscopy. Images were deconvolved with a blind deconvolution algorithm using the Huygens Essential program suite (SVI, Hilversum, The Netherlands).

Freezing of cells. Disks 2 mm in diameter, were cut out of the petriPERM dishes (Sartorius) with an ophthalmologic punch, transferred into hexadecane (Studer et al., 1989) prior to being placed into an aluminum chamber with a central cavity (height, 50 µm; diameter, 2 mm). The sandwich was then placed in the holder of the high-pressure freezer (EM HPM; Wohlwend Engineering, Sennwald, Switzerland) and immediately frozen. Cells grown on sapphire disks were frozen in a similar manner in a HPM010 freezer (BAL-TEC Inc., Balzers, Liechtenstein) as described in detail previously (Wild et al., 2002). The frozen samples were stored in liquid nitrogen until use.

Cryo-FESEM. The sandwich with the cells on the petriPERM disks within the aluminum planchets were clamped in a special holder (Walther and Müller, 1997) and cryofractured in a freeze-etching machine (BAF 300, BAL-TEC, Balzers, Liechtenstein) by removing one aluminum platelet with the microtome (temperature –110°C, vacuum about 2 x 10^6 to 7 x 10^6 mPa). After 2 min of etching (sublimation of some water at the fracture face), the sample was double-layer coated (Walther et al., 1995), by electron beam evaporation with 3 nm of platinum-carbon from an angle of 45° and about 8 nm of carbon perpendicularly. The frozen sample was then mounted on a cryoholder (Gatan, Pleasanton, CA, USA) and transferred into the microscope (S-5200 FESEM; Hitachi, Tokyo, Japan). The whole center of the punched-out petriPERM disk (about 1 mm^2) was analyzed at a temperature of –130°C and an acceleration voltage of 10 kV. Micrographs were recorded digitally using the back-scattered electron signal (Walther et al., 1995).

LTEM. Frozen cells grown on sapphire disks were transferred into a freeze-substitution unit (FS 7500, Boeckeler Instruments, Tucson, Arizona, USA) precooled to –88°C for substitution with acetone and subsequent fixation with 0.25% glutaraldehyde and 0.5% osmium tetroxide at temperatures between –30°C and 0°C as described previously in detail (Wild, 2001) and embedded in Epon. Sections (50 to 60 nm thick) were analyzed in a TEM (CM12; Philips, Eindhoven, The Netherlands) equipped with a slow scan charge-coupled device camera (Gatan) at an acceleration voltage of 100 kV.

CTEM. Cells grown on sapphire disks were fixed in 2.5% glutaraldehyde in 0.1 mM Na-K phosphate (pH 7.4) at 4°C for 1 h, postfixed in 1% osmium tetroxide in 0.1 mM Na-K phosphate (pH 7.4) at 4°C for 1 h, and dehydrated in acetone, followed by embedding, polymerization, and sectioning. To identify fluorescence signals, rBoHV-1-GFP-VP26-infected cells grown on carbon-coated sapphire disks were fixed with paraformaldehyd and examined by light microscopy with the areas of interest photographed and marked on the carbon film. After post-fixation with glutaraldehyde and osmium tetroxide and processing for TEM were then cut from the area of interest, and cells identified at low magnification.

Results

Virus multiplication and fate of cells. Cells infected with rBoHV-1-GFP-VP26 (MOI, 0.5) emitted faint to strong signals 28 h postinfection as detected by confocal microscopy (data not shown). To monitor the fate of infected cells, we chose cells emitting light signals at 28 h of infection for time-lapse microscopy in intervals of 6 min up to 42 h. Emission of signals enhanced drastically in a given cell first as diffusely distributed spots and later as bright patches (Fig. 1A). Correlative CTEM revealed that the finely dispersed signals were derived from capsids (Fig. 1B) or virions and that the bright patchy signals were derived from structures referred to as inclusion bodies, comprising accumulated GFP-VP26 (data not shown). Some cells disappeared rapidly in the course of incubation, whereas others remained intact for hours despite of accumulation of vast fluorescent material. Although integrity of membranous compartments was lost to various extents in cells shown to be intact or healthy by light microscopy, intact plasma membranes and fragmented nuclei or nuclei with peripheral chromatin condensation, capsids, and membranous inclusions could be seen (Fig. 1B). Deconvolution of DAPI-stained nuclei of uninfected cells revealed disk-like structures with long diameters of more than 18 μm and short diameters of only 2.5 μm (Fig. 1C). The chromatin of infected cells turned out to be very irregular, with protrusions and depressions (Fig. 1D). The longest diameter of nuclei was 14 to 15 μm, the shortest was up to 3.5 μm. To get an idea of size differences, we estimated the nuclear surface area from DAPI-stained DNA on the basis of the means of the long and short diameters, assuming that the nuclear surface was smooth. In doing so, the nuclear surface area was approximately 130 μm^2 in cells chosen at random at the beginning of incubation and about 160 μm^2 in cells chosen at random that had been incubated for 42 h (that is about 14 h of signal generation). The true surface of irregularly shaped nuclei, however, will certainly be larger than that obtained by this simple calculation, implying that the nuclear surface increases in the course of viral multiplication.

Nuclear Surface. To examine the nuclear surface by cryo-FESEM, we used cells infected with BoHV-1 at an MOI of 10 and incubated for 7 and 12 h for rapid freezing under high pressure, subsequent freeze-etching, unidirectional shadowing with platinum, and immediate

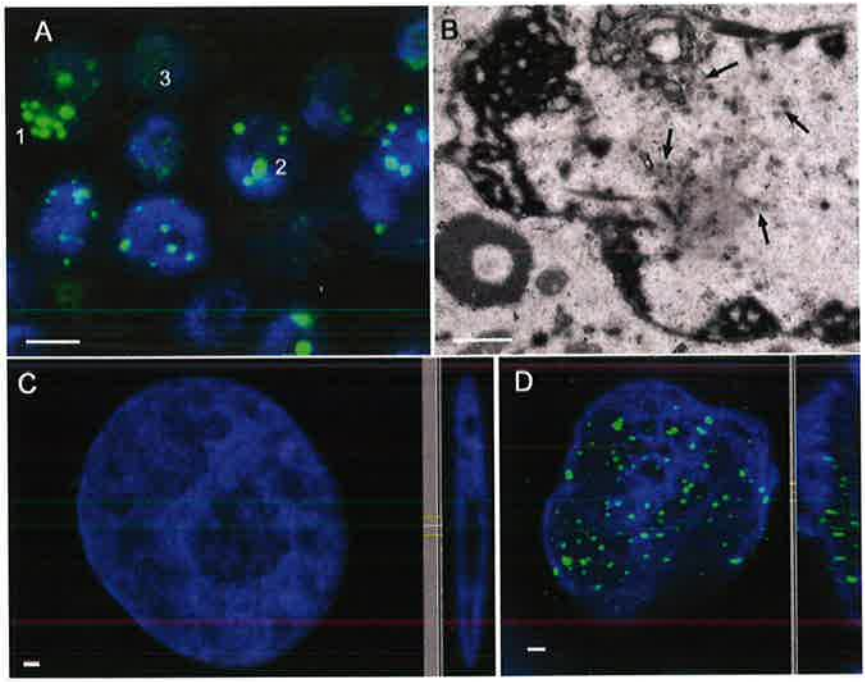

Fig. 1. **MDBK cells infected with recombinant BoHV-1 (MOI, 0.5) expressing the GFP-VP26 fusion protein.** (A) Cells after 42 h of incubation, fixation and DAPI staining. The intense signals in cell 1 and 2 correspond to the accumulation of capsids and/or virions embedded in protein within nucleus and cytoplasm (1) or only within nucleus (2). Nuclear fragments and membranes, despite lost integrity, were recognized by CTEM. (B) Micrograph obtained by CTEM from cell 3 with only faint fluorescence signal. The nucleus bears single capsids (arrows), capsids in small clusters, and membranous inclusions. Chromatin is condensed at the periphery, indicating apoptosis, and the nuclear membrane is intact. (C) Deconvolution of the nucleus after fixation and DAPI staining of a noninfected cell. The disk-like nucleus has diameters of 18.5, 15, and 2.5 μm, and a calculated surface of 133 μm². (D) Deconvolution of a nucleus after fixation and DAPI staining of an infected cell incubated for 42 h. The disk-like irregularly shaped nucleus with the short diameter of about 3.5 μm contains irregularly distributed chromatin and accumulation of capsids. The surface is calculated to be approximately 160 μm². Bars, 10 μm (A); 1 μm (B to D).

examination. Infected cells incubated for 7 h had nuclei with numerous nuclear pores containing pore complexes, similar to those found in uninfected cells, of about 120 nm in diameter (Fig. 2A and B). Some of the nuclear pores were enlarged up to 400 nm as confirmed by LTEM (Fig. 2C to H). In a few cells, many dome-like structures measuring 140

to 190 nm protruded into the perinuclear space. Size and shape make these protrusions very likely to be capsids at different stages of budding, as shown below by LTEM. At 12 h of incubation, nuclei were found to contain not a single nuclear pore. Instead, there were budding capsids with indentations of 50 to 180 nm, some containing budding capsids, and bulges of up to 500 nm (Fig. 3A and B). The indentations most probably represent the result of the budding process immediately after completion (Fig. 4E). Bulges of various sizes, some of them broken away during fracturing, were found by LTEM to be due to dilation of nuclear pores through which nuclear material containing capsids had projected into the cytoplasm (Fig. 3C to F). The border between nuclear material and cytoplasm was always distinct, indicating that the two compartments did not merge. Apart from impaired nuclear pores, the nuclear surface was intact (Fig. 3C). Impaired nuclear pores were not found in uninfected cells. The number and degree of dilation, size of bulges, and number of capsids within the escaping nuclear material and cytoplasmic matrix increased drastically from 7 to 12 h of incubation.

Nuclear membranes. For investigation cell membranes involved in envelopment, cells infected at an MOI or 1, 5, or 10 were incubated from 3 to 40 h. Cells were arrested by high-pressure freezing, freeze substituted (LTEM), and embedded in such a way that sections could be cut parallel to the smooth surface obtained by removing the sapphire disk. To determine the nature of the gaps in the nuclear membranes, we chose sections cut perpendicularly to the nuclear surface. Nuclei of both infected and uninfected cells clearly revealed intact 100-nm-wide nuclear pores (Fig. 2C) with a distinct central layer corresponding to the central ring and a cloudy layer corresponding to the cytoplasmic ring and filaments of the nuclear pore complex (Pante and Aebi, 1996). Gaps in the nuclear membrane measured 140 to 400 nm early in infection (defined as an MOI of 0.5 at 24 to 28 h, an MOI of 1 and 5 at 12 to 16 h, and an MOI of 10 at 3 to 5 h) (Fig. 2D to H), and up to 1900 nm late in infection (defined as an MOI of 0.5 at 40 to 42 h, an MOI of 1 and 5 at 33 to 40 h, and an MOI of 10 at 12 to 14 h) (Fig. 3D to F). Dilations of 140 to 200 nm were already found in cells incubated for only 5 h at an MOI of 10. They were distinctly bordered by the inner nuclear membrane continuing into the outer nuclear membrane and thus were assumed to represent impaired nuclear pores lacking nuclear pore complex structures. Capsids were often present in front of (Fig. 2G and 3D), within (Fig. 3F), and close to (Fig. 2D and H and 3D) impaired nuclear pores.

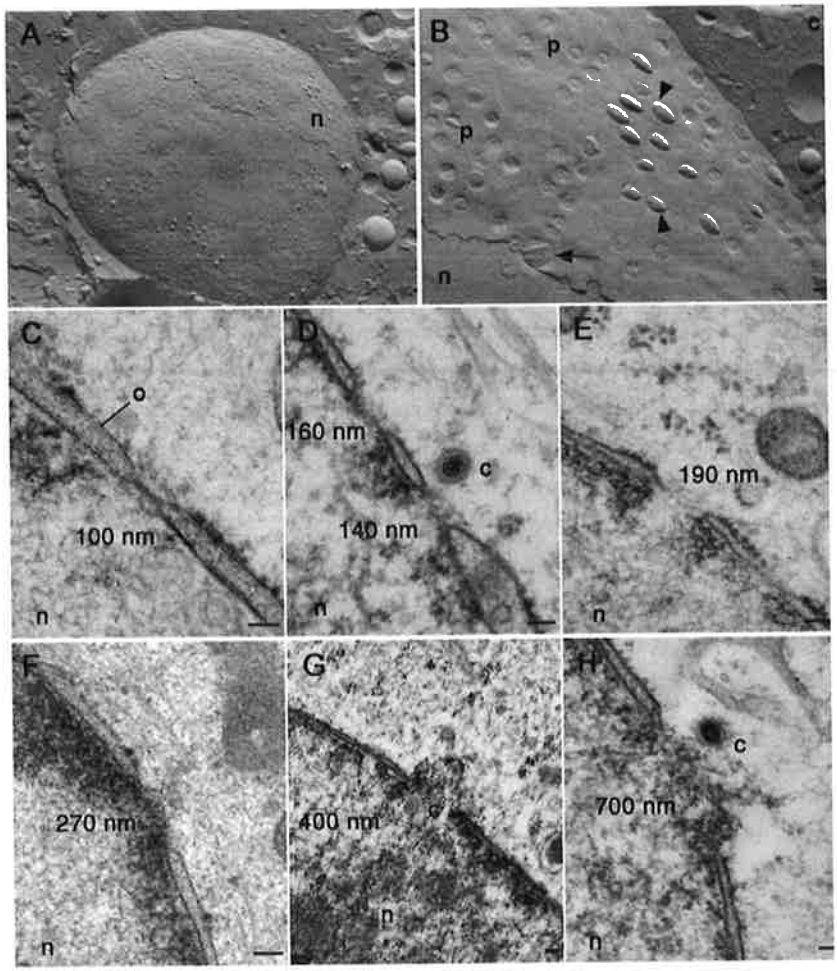

Fig. 2. **Nuclear surface of MDBK cells early in infection with BoHV-1 at an MOI of 10 (A to D), 5 (E and H), or 1 (F and G).** n, nucleus. (A and B) Cryo-FESEM images reveal intact nuclear pores (p), budding capsids (arrowheads) and a dilated nuclear pore (arrow). (C to H) TEM images of sections cut perpendicular to the nuclear surface. (C) Intact nuclear pore with distinct central density corresponding to the central plug of the nuclear pore complex and distinctly visible inner and outer (o) nuclear membrane. (D to H) The diameter of nuclear pores, defined by the border where the intact inner nuclear membrane merges with the outer nuclear membrane, is enlarged from 140 up to 700 nm. Nuclear content is within the border delineated by nuclear membranes in dilated pores up to about 200 nm (E), arches through impaired pores with diameters of about 300 nm (F), and bulges into the cytoplasm through more severely dilated pores (G and H). Capsids (c) are either just outside (D) but still in contact (H) with nuclear material or within nuclear material (G), suggesting that they are released via impaired nuclear pores. Bars, 1 μm (A); 100 nm (B to H).

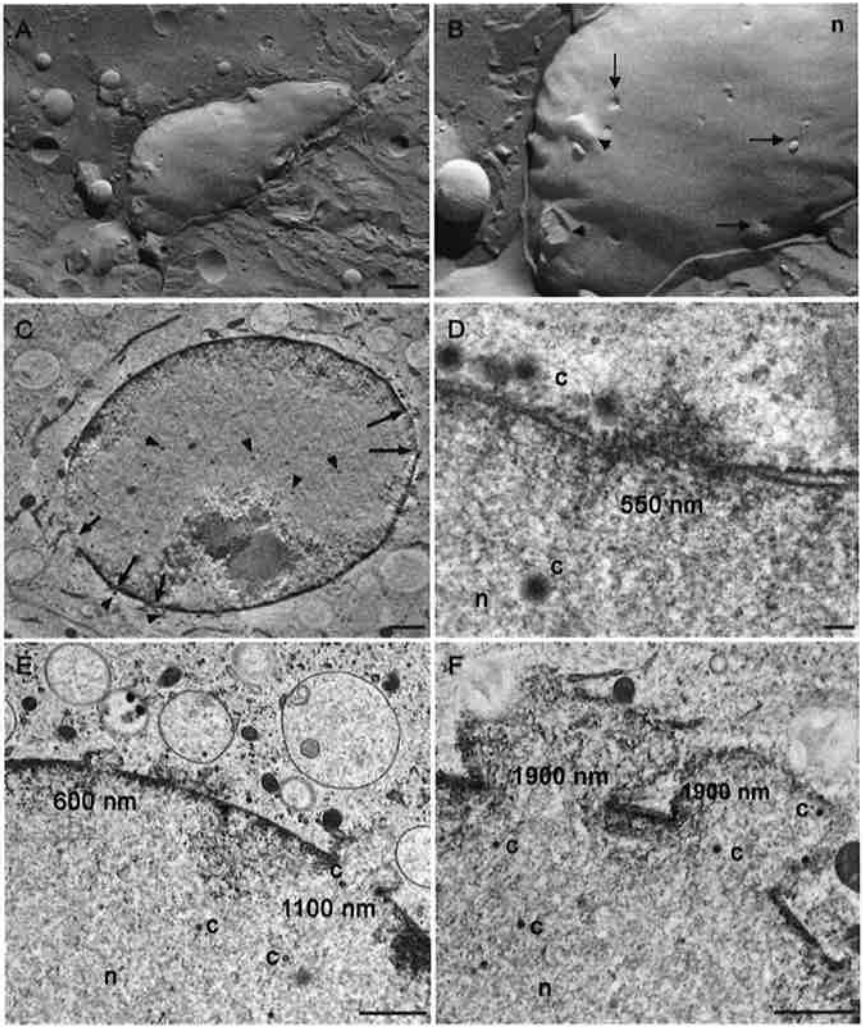

Fig. 3. **The nuclear surface of MDBK cells late in infection with BoHV-1 at an MOI of 10 (A, B, and D), 5 (C and E), or 1 (F).** (A and B) Cryo-FESEM images reveal a few budding capsids or holes, most probably indicating that budding has already been completed (arrows). Nuclear pores are not visible. Instead, there are protrusions of nuclear matrix (arrowheads); one broke away during freeze-etching. (C to F) LTEM images of intact nuclei with distinctly defined nuclear pores that are dilated up to 1,900 nm. Nuclear matrix containing capsids (c) protrudes into the cytoplasm but does not merge with it. Bars, 1 μm (A to C, E, and F); 100 nm (D).

LTEM also revealed capsids at various stages of budding through the inner nuclear membrane (Fig. 4A, C, and D). The capsid in intimate contact to the inner nuclear membrane (Fig. 4C) is considered to be in an initial phase of budding pushing the membrane slightly into the perinuclear space. In an intermediate phase (Fig. 4D), both the inner and outer nuclear

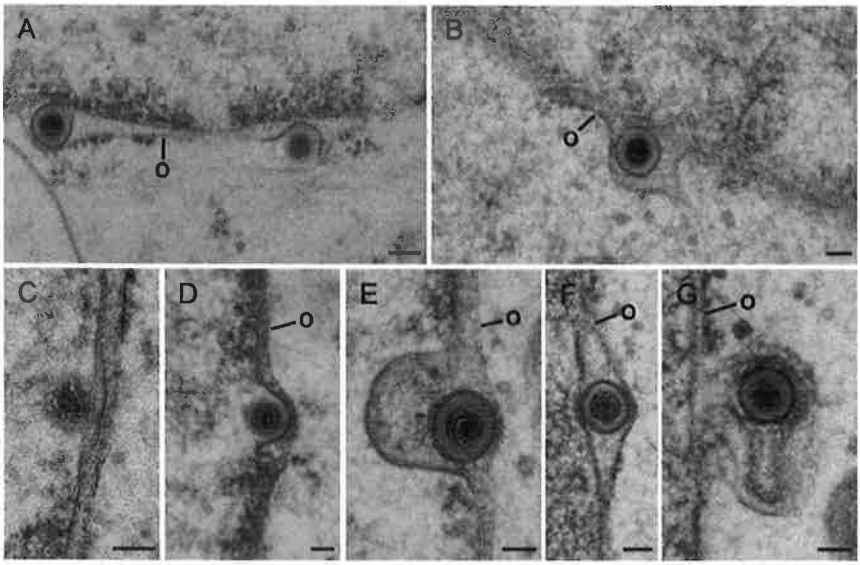

Fig. 4. **The inner nuclear membrane and the perinuclear space early in infection at an MOI of 1 (A and B) or 10 (C to G).** (A) At the left is a capsid close to completion of budding at the inner nuclear membrane containing a dense envelope that is in close apposition to the outer nuclear membrane (o). At the right is a capsid with about three-fourths of the entire envelope acting as part of the outer nuclear membrane. The tegument is only present between the capsid and the membrane, which is turned almost 180° at one side. This process is described in the legend to Fig. 5. (B) Virion with a dense envelope in the perinuclear space that continues into RER cisterna. (C) Initial phase of a budding capsid at the inner nuclear membrane. Slight thickening of the nuclear leaflet and the course of the membrane underneath the capsid are demonstrated because of underfocusing. (D) Intermediate phase of budding with distinct thickening of the inner nuclear membrane that is sharply bent at one side. Some tegument is deposited around the capsid. To form an enveloped particle, the membrane must be pulled into the nucleus behind the capsid for fusion. (E) Virion within the perinuclear space probably shortly after budding was completed because of the large indentation of the inner nuclear membrane. (F) Virion within the perinuclear space with a dense layer at the envelope that is in close apposition to both the inner and the outer nuclear membrane. (G) Virion within a cisterna continuous to the perinuclear space, implying an intracisternal transport. Bars, 100 nm.

membranes are pushed far into the cytoplasm. The inner nuclear membrane is thickened by a dense material that reaches exactly from one site where it turns into the perinuclear space to the other site. The membrane needs to be pulled at this turn behind the capsid for fusion to occur, which results in a fully enveloped virion and a deep indentation (Fig. 4E) also recognizable by cryo-FESEM (Fig. 3B). Fully enveloped virions were found in the perinuclear space (Fig. 4F), in conjunctions to associate cisternae (Fig. 4B) and within associated RER cisternae (Fig. 4G). They always exhibited a thick dense envelope that was nearly always in close contact to both the inner and outer nuclear membrane.

Table 1. Distribution of virus particles in 20 cellular profiles chosen at random in ultrathin sections of MDBK cells incubated for 16, 20, and 40 h with BoHV-1 at an MOI of 1[a]

h	Nca	Budding			Packaging		Wrapping			I-NP
		IM	OM	PS	Go	Vac1	Cca	Go	Vac2	
16	180	1	2	3	22	62	51	20	41	7
20	410	0	1	2	27	137	93	23	35	6
40	470	1	0	1	2	23	310	35	17	11

[a] Nca, Nuclear capsids; IM, inner nuclear membrane; OM, outer nuclear membrane; PS: perinuclear space and associated RER; Go, Golgi membranes; Vac1, large vacuoles (Fig. 3B to E) (Wild et al., 2002); Cca, cytoplasmic capsids; Vac2, small vacuoles (Fig. 6E); I-NP, impaired nuclear pores. Note that numbers of viral particles and their distribution vary considerably among cells.

Although one might conclude from Fig. 2A that budding at the inner nuclear membrane is a frequently occurring event, we very rarely found budding by LTEM (Table 1). Indeed, we have only a few more micrographs convincingly demonstrating budding capsids at the inner nuclear membrane. Interestingly, we found more capsid-membrane interaction at the outer nuclear membrane than at the inner nuclear membrane. Because of the widely accepted theory that the envelope derived by budding through the inner nuclear membrane fuses with the outer nuclear membrane to release the capsid into the cytoplasm, we paid especial attention to this process. However, the trial designed to test our findings with the fusion theory failed. LTEM rather revealed budding events than fusion events. The capsids in close contact to the outer nuclear membrane, which is slightly pushed toward the perinuclear space (Fig. 5B to D) and slightly thickened (Fig. 5C and D), are considered more likely to be nearing the budding stage

than the result of fusion, because the relation of capsids to the outer nuclear membranes exactly resembles that to the inner nuclear membrane (Figs. 4C and 5C). The intermediate states of the process taking place at the outer nuclear membrane (Fig. 5E to G) are characterized by the thickened membrane bending deep into the perinuclear space, exactly as such bending is caused by budding capsids at the inner nuclear membrane. The thickened membrane reaches from one side, where the outer nuclear membrane turns into the perinuclear space to the other side. The length of the thickened membrane equals about two-third of the final entire envelope, as does the thickening of the inner nuclear membrane. The only difference from the inner nuclear membrane is that the inner membrane turns next to the nucleus are less than 90° or even zero (Fig. 5F), whereas it is 180° on the opposite side, so that the thickened envelope runs in parallel with the outer nuclear membrane for about 100 nm. To the best of our knowledge, this cannot happen in the course of fusion. We, hence, consider the data shown in Fig. 5E to 5G to represent intermediate phases of budding. Consequently, capsids (Fig. 5H and I) are assumed to be close to completion of budding into the perinuclear space at a narrow angle in relation to the outer nuclear membrane, which also makes the typical turn of 180°. Interestingly, the capsid shown in Fig. 5H buds at the site where the perinuclear space continues into an associated cisterna, the envelope is only four-fifths of its final length, and tegument is lacking at the site where the membrane needs to fuse for fission. In the results shown in Fig. 5I, tegumentation is completed, and the membrane is close to fusion or even in fusion, which results in fission of the enveloped virion from the outer nuclear membrane. There is no indication of fusion of the thickened envelope with the outer nuclear membrane; at this site, fusion would be expected if it occurred.

Golgi complex. Hundreds of capsids were found in the cytoplasm (Table 1), many of them approaching Golgi membranes (Fig. 6A) or being enveloped by Golgi membranes. Capsids approached membranes at any site of the Golgi complex, most frequently, however, at the site closest to the nucleus (Fig. 6A, B, and D). Envelopment by Golgi membranes, termed wrapping, is analogous to budding at nuclear membranes. Budding starts by bending of the membrane at site of close contact into Golgi cisterna and concomitant deposition of dense substance at the bending membrane (Fig. 6B). Subsequently, tegument is deposited between membrane and capsid, and bending proceeds, involving the entire membrane of the cisterna (Fig. 6C). To form an envelope, the membrane must be pulled behind the capsid and tegument for fusion that leads to an enveloped virion by fission from the Golgi membrane (Fig. 6D).

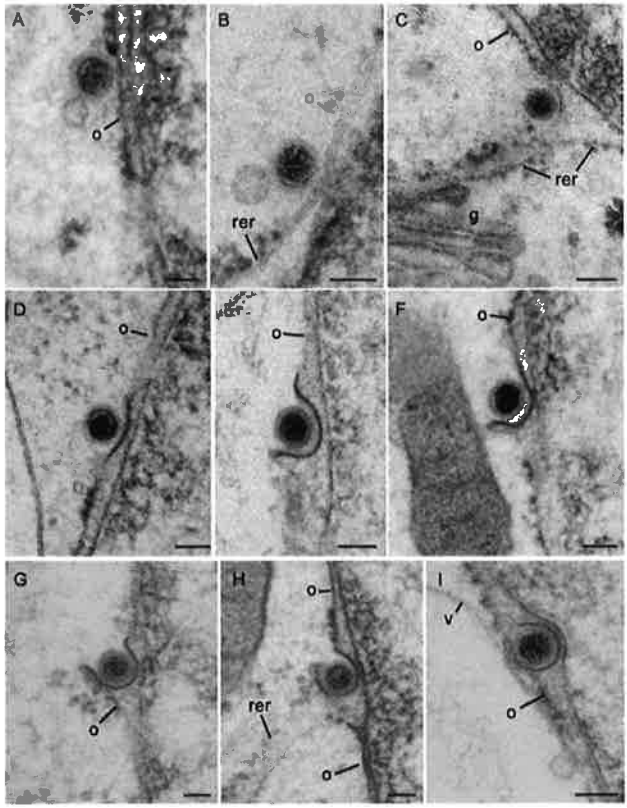

Fig. 5. **The outer nuclear membrane and the perinuclear space early in infection, at an advanced stage of infection (defined as MOIs of 1 and 5 at 20 to 23 h, or an MOI of 10 at 7 to 8 h), and late in infection; an MOI of 5 (A to C), 1 (D, F, and H), or 10 (E, G, and I).** (A) Capsid in intimate contact to the outer nuclear membrane (o) at a site free of ribosomes next to a dilated nuclear pore. (B) Capsid in intimate contact to the outer nuclear membrane that is slightly bent toward the perinuclear space. (C) Capsid in intimate contact to the outer nuclear membrane that is thickened and slightly bent toward the perinuclear space. The perinuclear space is continuous to the RER (rer) in contact with a Golgi membrane (g). (D) Capsid in intimate contact to the outer nuclear membrane distinctly thickened and bent. There is little or no tegument between the capsid and membrane. Panels A to D are considered to represent initial phases of budding at the outer nuclear membrane. (E to G) Capsids at the outer nuclear membrane that is markedly thickened and bent into the perinuclear space. The membrane turns close to 180° at one side of the capsid that is clearly separated from the membrane by tegument. These are considered to be intermediate phases of budding. (H) Capsid at the thickened outer nuclear membrane that bends deeply into the perinuclear space just at the site where it continuous into an RER cisterna. The outer nuclear membrane makes also a turn of close to 180° at one side of the capsid. The space between the membrane and the capsid is filled by tegument. (I) Capsid within the perinuclear space surrounded by tegument and the outer nuclear membrane that turns 180° at the cytoplasmic side. v, membrane of an empty vacuole. Bars, 100 nm.

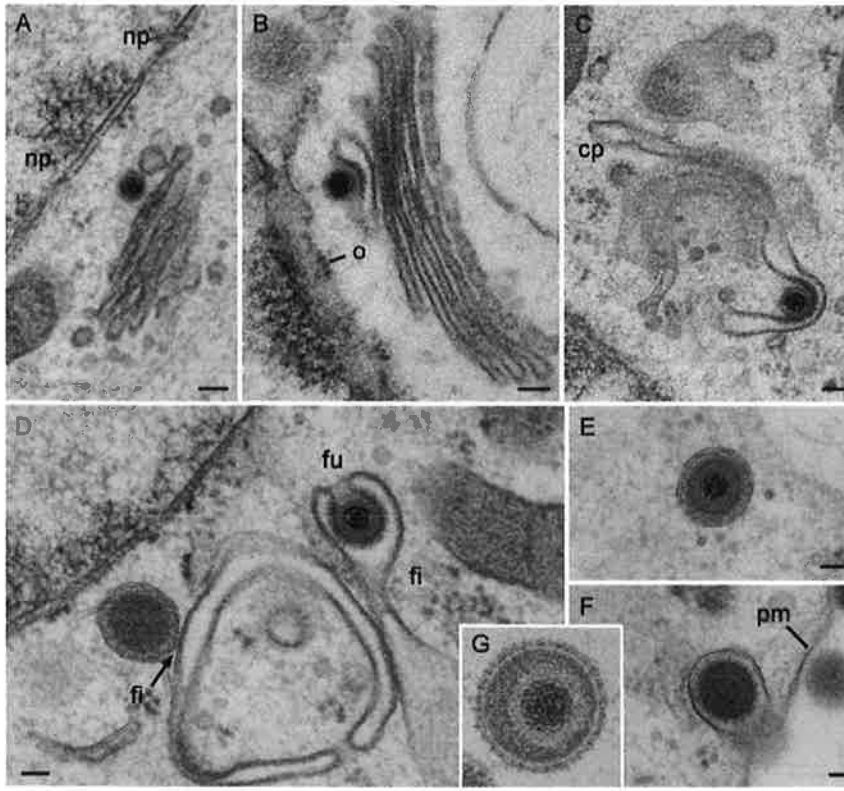

Fig. 6. **Budding capsids at Golgi membranes and formation of transport vacuoles (wrapping) at advanced and late stages of infection, at an MOI of 1 (F), 5 (B, C, and E), or 10 (D and G).** (A) Capsid approaching a Golgi membrane close to two dilated nuclear pores (np). (B) Capsid less than 100 nm from the outer nuclear membrane (o), in initial phase of budding with slight bending and thickening of the membrane in contact with the capsid. (C) Intermediate phase of budding with thickening of the membrane and little deposition of tegument. cp, coated pit. (D) Late phase of budding with tegument surrounding the capsid, clearly visible spikes, and start of fusion (fu) of Golgi membranes at the basal pole; wrapping is initiated by initation of fission (fi; right) but is close to completion at the left (fi; arrow) forming a spherical vacuole containing the virion (tangentially sectioned) and a dense substance completely hiding the spikes. (E) Spherical transport vacuole comprising a virion and dense substance-hiding spikes. (F) The membrane of a transport vacuole with a tangentially sectioned virion fuses with the plasma membrane (pm) releasing virion and antifusion proteins into the extracellular space. (G) Virion in the extra cellular space, comprising capsid with dense core, tegument, and envelope with spikes. Bars, 100 nm.

Because the entire cisterna is involved, a vacuole is formed that needs to be detached (Fig. 6D). Spikes are distinctly visible in the process of envelopment. The vacuoles containing the fully enveloped virion are spheres with the virion in their center. The concentric space between envelope and vacuolar membrane is occupied by dense substance that seems to cover spikes (Fig. 6D to F). The vacuoles transport the virions toward the cell periphery, where the vacuolar membrane fuses with the plasma membrane (Fig. 6F), and the virion with its envelope full of spikes is released into the extra cellular space (Fig. 6G). Apart from envelopment of approaching capsids from the cytoplasm, the Golgi complex is involved in packaging of virions, leading to entirely different vacuoles containing one or several virions with envelopes exhibiting distinct spikes (Wild et al., 2002). Golgi complexes dramatically increased after 5 h of incubation at an MOI of 10, and at 12 to 16 h at MOI of 1 and 5, compared to control cells. The Golgi complex, however, often consisted of only one or two cisternae or even of remnants in cells late in infection.

Discussion

Infected MDBK cells produced and accumulated GFP-labeled capsids, virions, and proteins for many hours and suddenly disintegrated within a few minutes. Disk-like nuclei of cells at the beginning of incubation turned into disk-like nuclei with slightly decreased long diameters, increased short diameters of about 40%, and highly irregular surface. In an attempt to investigate the nuclear surface early in infection, we discovered distortions of nuclear pores involving the loss of nuclear pore complexes and enlargement of pore diameters from 140 nm up to 1,900 nm, while the nuclear membranes remained intact. The diameter of isolated nuclear pore complexes is 125 nm (Pante and Aebi, 1996). We measured diameters of intact pores by LTEM as 100 to 110 nm. The discrepancy is probably due to section thickness that does not allow precise measurements. At the site of both intact and impaired nuclear pores, the inner nuclear membrane turns into the outer nuclear membrane. Impairment of nuclear pores was obvious also by CTEM (data not shown), and nicely documented in nuclei late in infection with simian agent 8 (Borchers and Oezel, 1993).

Impairment of nuclear pores raises the question as to whether or not it plays an essential role in rapid cell disintegration. To answer this is a difficult task and impossible on the basis of our data. The facts, however, that nuclear pore impairment had already occurred 7 h after infection in cells that were active in viral envelopment and had all other cellular structures intact and that cells accumulating virus remained active in terms of viral production for many

hours and then disintegrated within a few minutes suggests that nuclear pore impairment per se is not responsible for cell death. The presence of capsids at sites of impaired nuclear pores suggests that capsids use these pores as gateways to the cytoplasm, as was supposed for simian agent 8 (Borchers and Oezel, 1993). This idea is supported by the low frequency of budding capsids encountered at the inner nuclear membrane and by the absence of any convincing fusion events at the outer nuclear membrane.

There may be two reasons for the low frequency of detectable budding capsids. Either the process is very rapid so that the chance to hit a capsid at the very moment of budding is low, or budding is a rare event. Fusion between influenza virus and liposomes is completed within 1 min (Kanaseki et al., 1997). Budding at the inner nuclear membrane involves thickening of the nuclear membrane, driving the capsid toward the perinuclear space, deposition of tegument, and fusion of the inner nuclear membrane so that the enveloped virion can be detached. Considering that the fusion process alone takes 1 min, the entire budding process will take much longer. Hence, one must assume that budding ought to be seen easily if the frequency of budding were high. Indeed, budding can be visualized by cryo-FESEM impressively early in infection (Fig. 2). Budding can also be demonstrated very easily at Golgi membranes at almost any point in infection when envelopment has started. Budding at Golgi membranes is a process very similar to budding at the inner nuclear membrane. It involves deposition of dense substance at the membrane, driving the capsid toward Golgi cisternae, deposition of tegument, and fusion of Golgi membranes so that the envelope can be detached, finally lying in a vacuole that is derived by fission of Golgi membranes. In addition, the concentric space between envelope and vacuolar membrane is filled with a dense substance, a finding that had been demonstrated as early as 1988 (Lycke et al., 1988). We assumed that this substance has the same function as the substance deposited at the membrane during budding at nuclear membranes. Although wrapping by Golgi membranes is slightly more complicated than budding at the nuclear membrane, the time required for wrapping is probably not much longer than that of budding, because fission of the envelope may be overlapped by fission of the transport vacuole. It is thus fair to assume that the chance of hitting a budding capsid at the inner nuclear membrane is equal to that of hitting one at Golgi membranes, provided the number of capsids is the same. Considering that the nucleus is hit in many more sections through a given cell than the Golgi complex, budding capsids at the inner nuclear membrane ought to be encountered very frequently. Since this is certainly not the case, it is reasonable to assume that budding at the inner nuclear membrane is a rare event.

If the fusion theory were correct, the number of budding capsids at the inner nuclear membrane would equal the number of envelope fusion with the outer nuclear membrane. Fusion certainly would be faster than budding, meaning that the chance of hitting an envelope in fusion is less than that of hitting a budding capsid. Interestingly, our results demonstrate that the number of capsids involved in the process at the outer nuclear membrane is higher than that at the inner nuclear membrane. To draw a final conclusion from this low numbers of events encountered with hundreds of cells is not possible. Together with the following consideration, however, this observation definitely does not support the fusion theory. The surface area of a virion with a diameter of 200 nm (Grunewald et al., 2003; Zhou et al., 1999) is 0.125 μm^2. The surface of 1,000 virions – a number reasonably assumed to be produced by one cell – equals the surface area of a sphere-like nucleus with a diameter of 6.3 µm or approximatively of a disk-like nucleus with axes of 18.5, 16 and 2.5 µm giving a surface of 133 μm^2 (Fig. 1). This means that the entire inner nuclear membrane would be inserted into the outer nuclear membrane, which in turn would need to be shifted to the inner nuclear membrane at the site they meet each other (at the nuclear pores) within a few hours. We do not know whether or not this shift is possible. However, it seems rather difficult because of the steady interference by the budding and the fusion processes. Furthermore, cell volume and cell surface area increased, although only slightly in the course of virus production, implying either additional membrane supplementation or augmentation of the surface area exclusively due to enlargement of nuclear pores. Another crucial point is the thickening of the membrane that becomes the viral envelope, as shown by many other authors (Baines et al., 1995; Borchers and Oezel, 1993; Church and Wilson, 1997; Fong et al., 1973; Gershon et al., 1994; Granzow et al., 1997; Lecatsas and Poste, 1973; Pol et al., 1991; Roller et al., 2000). What is the role of the membrane thickening? Why would it disappear immediately after fusion with the outer nuclear membrane? And why is it present in virions within the perinuclear space and even in its associated cisternae? There is no answer to this from the standpoint of the fusion theory.

A plausible significance of this membrane thickened, due to deposition of a dense substance, a protein(s) of as-yet-unknown nature, is to prevent the envelope from fusion with the outer nuclear membrane, the inner nuclear membrane, and the membranes of those cisternae through which the virion is transported. If virions can be transported within the perinuclear space into associated cisternae, and there are many indications that they do (Borchers and Oezel, 1993; Browne et al., 1996; Card et al., 1993; Di Lazzaro et al., 1995; Fong et al., 1973;

Gershon et al., 1973; Gershon et al., 1994; Granzow et al., 1997; Radsak et al., 1996; Roller et al., 2000; Schwartz and Roizman, 1969; Whealy et al., 1991), the viral envelope must be unable to fuse. Alternatively, if the viral envelope could fuse with the outer nuclear membrane, it could probably also fuse with the inner nuclear membrane; certainly, it cannot be transported within spaces where those membranes need to be in close contact with the envelope simply, because of size differences. The theoretical considerations are well supported by the morphology of the process taking place at the outer nuclear membrane. (i) Capsids in close contact with the membrane, which may be slightly bent into the perinuclear space, are very difficult to imagine once released from the perinuclear space by fusion. (ii) Membranes in fusion do not make 180° turns (Fig. 5). Fusion of membranes starts by close apposition, followed by formation of fusion stalks and finally of fusion pores (Kozlovsky et al., 2002; May, 2002; Melikyan and Chernomordik, 1997), a process of high symmetry that can be visualized by electron microscopy employing cryotechniques (Kanaseki et al., 1997). (iii) The envelope-membrane interaction at the outer nuclear membrane shows high identity with the process of budding capsids at the inner nuclear membrane. The only difference between the process at the inner and the outer nuclear membrane is the angle between the axis of capsid movement and the nuclear periphery. This angle is about 90° at the inner membrane and 30 to 40° at the outer nuclear membrane. Budding capsids at the inner nuclear membrane are driven toward the outer nuclear membrane that can give way by projecting into the cytoplasm. Capsids budding at the outer nuclear membrane must be driven at a narrow angle into the perinuclear space because the nucleus is solid compared to the cytoplasmic matrix. Indications for budding at the outer nuclear membrane were also published for herpes simplex virus 1 (Lopez-Iglesias and Puvion-Dutilleul, 1988) and simian agent 8 (Borchers and Oezel, 1993).

If capsids are not released into the cytoplasm by fusion, what is the fate of the virions derived by budding at the nuclear membranes? As mentioned above, they are transported into associated RER cisternae. RER cisternae have been shown to continue into Golgi cisternae (O'Donnell et al., 1988; Wang et al., 2001; Wild et al., 2002). Consequently, virions can be transported directly from the perinuclear space via RER cisternae into Golgi cisternae (Wild et al., 2002). There, they are packaged into transport vacuoles that are entirely different from transport vacuoles derived from wrapping. Vacuoles originating from packaging are of various sizes containing one or more virions with an envelope missing the dense layer. Instead, spikes are clearly apparent; the space between viral envelopes and vacuolar

membrane is not occupied by the dense material, as in concentric vacuoles derived by wrapping. This substance is assumed to prevent the viral envelope from fusion with the vacuolar membrane. It thus is speculated that it is the same substance that is deposited at the viral envelope in the course of budding at nuclear membranes. The same substance might be present also in large vacuoles derived by packaging though at lower concentrations.

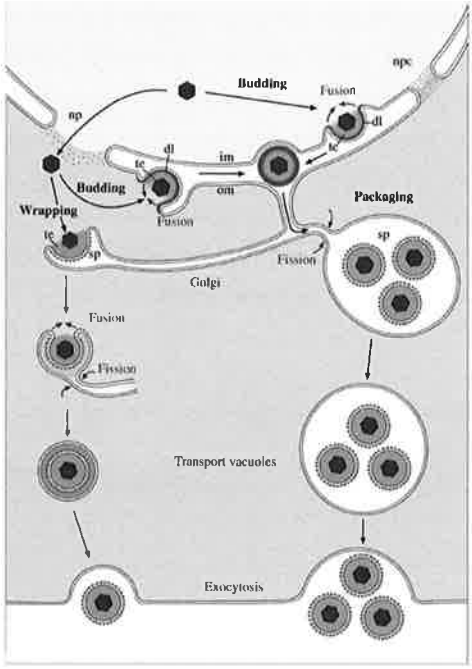

Fig. 7. **Schematic drawing of the dual pathway of BoHV-1 envelopment.** In the wrapping pathway, capsids leave the nucleus via impaired nuclear pores (np) and approach Golgi membranes from the cytoplasmic side, inducing budding. Since the entire cistern is involved, a sphere-like structure comprising two membranes arises. The inner membrane becomes the viral envelope and the outer becomes the vacuolar membrane. Fusion of the envelope with the vacuolar membrane is prevented by antifusion proteins at high concentrations. In the packaging pathway, capsids bud through the inner or outer nuclear membrane and are transported from the perinuclear space via RER cisternae into Golgi cisternae for packaging into transport vacuoles of various sizes containing one or more virions. The fusion of viral envelope with cell membranes is prevented by proteins of as-yet unknown nature localized at the viral envelope. The antifusion proteins are cleft off in Golgi cisternae but remain there (possibly together with additional proteins at low concentration) to prevent fusion of viral envelopes with the membrane of large transport vacuoles. im, inner nuclear membrane; om, outer nuclear membrane; npc, nuclear pore complex; dl, dense layer (antifusion proetein); sp, spikes; te, tegument.

Taken together, the two sorts of transport vacuoles imply diverse pathways of capsids through the Golgi complex (Fig. 7). One pathway involves budding at Golgi membranes, resulting in a centrally located virion within a small vacuole containing antifusion protein at high concentrations. This pathway is designated the wrapping pathway. In the other pathway, virions, whose envelope and tegument originated by budding at nuclear membranes, are covered by an antifusion protein and hence can be transported within the perinuclear space and associated RER cisternae into Golgi cisternae for packaging into large vacuoles. The antifusion protein covering the viral surface is cleft off, possibly remaining together with additional antifusion proteins within vacuoles. This pathway is designated the packaging pathway. Packaging and wrapping may take place simultaneously at the same Golgi complex (Wild et al., 2002). The number of capsids that bud at the inner nuclear membrane is probably low and higher early in infection. Capsids approaching Golgi membranes from the cytoplasmic side escape from the nucleus via impaired nuclear pores. Capsids have the ability to induce budding at the nuclear side and the cytoplasmic side of cell membranes, provided the machinery necessary for the budding process is present.

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Chapter 3

Envelopment of Herpes Simplex Virus 1 Capsids Requires Glycoprotein K

Abstract

The formation of infectious herpes simplex virus 1 (HSV-1) depends on a budding process of capsids through cellular membranes to acquire tegument and a viral envelope. The UL53 gene of HSV-1 encodes the glycoprotein K (gK) that is essential for virus replication. To investigate the role of gK during the egress pathway, we generated a gK deficient mutant (CSΔgK). No infectious virus particles were formed in CSΔgK infected cells. Formation of plaques could be restored on a stable cell line expressing gK from AAV2 p5 promoter. The p5 promoter was demonstrated to be inducible upon HSV-1 infection. Electron microscopy of CSΔgK infected Vero cells revealed that the amount of intracellular capsids produced during replication was similar to wild-type, but the majority of CSΔgK particles (up to 98%) were distributed throughout the cytoplasm as naked capsids. The lack of virus particles within the perinuclear space in the absence of gK and the missing integrity of nuclear envelope suggests that capsids exit the nucleus via impaired nuclear envelope to gain access to the cytoplasm. In the absence of gK, envelopment of capsids was blocked at both the nuclear and Golgi membranes. Consistently, even late in infection (up to 48 h pi) the Golgi complex was not fragmented in CSΔgK infected cells in contrast to HSV-1 infected cells, implying that Golgi membranes were not consumed for envelopment in the absence of gK. Our data suggest that gK is responsible for capsid budding at any cellular membranes.

Introduction

Herpes simplex virus type 1 (HSV-1) virion comprises four structural elements: the DNA-containing core within the icosahedral capsid, the tegument surrounding the capsid, and the envelope in which viral glycoproteins are embedded (Wildy et al., 1960). To date 12 glycoproteins have been identified in HSV-1 playing a role in cell attachment, cell tropism and virus morphogenesis. Five of HSV-1 glycoproteins - gB, gD, gH, gL and gK - have been shown to be indispensable for virus replication (Cai et al., 1987; Forrester et al., 1992; Jayachandra et al., 1997; Ligas and Johnson, 1988; Roop et al., 1993). gB, gD, gH and gL are required for cell entry, whereas gK is essential for egress (Browne et al., 2001; Hutchinson and Johnson, 1995; Pertel et al., 2001; Turner et al., 1998). Mutants lacking one of the glycoproteins needed for entry were able to produce enveloped particles containing processed glycoproteins (Cai et al., 1987; Forrester et al., 1992; Johnson and Ligas, 1988; Roop et al., 1993).

gK is a highly hydrophobic protein of 338 amino acids which traverse membranes several times (Foster et al., 2003a; Mo and Holland, 1997). It contains a cleavable 30-amino acid signal sequence at its amine terminus and two *N*-linked glycosylation sites (Pogue-Geile and Spear, 1987; Ramaswamy and Holland, 1992). gK homologs are almost as highly conserved as the gB homologs among alphaherpesvirus glycoproteins (DebRoy, 1990; Foster and Kousoulas, 1999; Klupp et al., 1998; McGeoch et al., 1991; Mo et al., 1999; Neubauer and Osterrieder, 2004). Transcription of UL53, which encodes for gK, depends on viral DNA synthesis, which is characteristic for members of the late kinetic class of HSV-1 genes (Debroy et al., 1985). *In vivo*, UL53 gene, among other genes, is associated with neurovirulence (Ben-Hur et al., 1996). Compared to other HSV-1 glycoproteins, HSV-1 infected cells express relatively low levels of gK. It localizes mainly in membranes of the nucleus, the endoplasmic reticulum and Golgi complex (Hutchinson et al., 1992; Hutchinson et al., 1993; Hutchinson et al., 1995). Cell surface expression of gK is dependent on the UL20 protein expression suggesting that UL20 protein directly interacts with gK (Avitabile et al., 2004; Foster et al., 2003a; Foster et al., 2004). Recently, it could be demonstrated that gK is a component of the virion (Foster et al., 2001). Single nucleotide substitution in UL53 gene can cause extensive virus induced cell fusion (Debroy et al., 1985; Dolter et al., 1994; Pogue-Geile and Spear, 1987). Syncytial mutations in UL53 gene are more frequently isolated than syncytial mutations in any other HSV-1 gene (Bond and Person, 1984; Bond et al., 1982; Read et al., 1980). However, these cell fusion phenotypes caused by mutated gK protein can

be suppressed by expressing wild-type gK protein (Hutchinson et al., 1993). Based on these findings, gK is suggested to suppress or regulate membrane fusion (Avitabile et al., 2004; Hutchinson et al., 1993; Hutchinson et al., 1995).

Initial attempts to delete the HSV-1 UL53 gene were unsuccessful, considering that the UL53 gene was essential for virus replication (Foster and Kousoulas, 1999; Hutchinson and Johnson, 1995). Infectious virus particles of gK deletion mutants could only be recovered from gK complementing cells. Vero cells and human fibroblast cells infected with gK deficient mutants produce very few extracellular virions and feature only singly infected cells (Foster and Kousoulas, 1999; Hutchinson and Johnson, 1995). In the absence of gK, translocation of infectious virions from the cytoplasm to the extracellular spaced is blocked and unenveloped capsids accumulate in the cytoplasm of infected cells (Hutchinson and Johnson, 1995; Jayachandra et al., 1997). Moreover, HSV-1 virions derived from cells in which gK are not expressed displayed a reduced capacity to enter cells and initiate early stages of virus replication (Foster et al., 2001). However, glycoprotein synthesis and cell surface expression of glycoproteins were not impaired in the absence of gK (Hutchinson and Johnson, 1995). Differences were described between the clinical strain HSV-1 (F) and the laboratory strain HSV-1 (KOS). Active replicating cells could partially complement gK deficient mutant of HSV-1 (KOS), whereas the replication efficiency of HSV-1 (F) gK deficient mutant remains low in log-phase cells (Jayachandra et al., 1997). This result suggests that even no apparent differences were noted between the parental F and KOS virus strain regarding their replication efficiency, multiple passages of viruses in the KOS background may have resulted in genetic differences and thus improved adaptation of the mutant virus for growth.

Mutant viruses that are deficient in gK expression have also been constructed in pseudorabies virus (PRV), equine herpesvirus type 1 (EHV-1) and varicella-zoster virus (VZV). gK of VZV, EHV-1 and PRV, like its homolog in HSV-1, were critical for viral replication (Klupp et al., 1998; Mo et al., 1999; Neubauer and Osterrieder, 2004). Infection with these gK deletion mutants resulted in singly infected cells or very small foci and substantial reduction in the production of infectious virions. In the absence of PRV gK, many capsids were found just underneath the plasma membrane, and thus, PRV gK was suggested to have a role in preventing immediate reentry of released virions (Klupp et al., 1998).

Interestingly, not only deletion but also overexpression of gK drastically influences HSV-1 replication (Foster et al., 2003b). The intracellular effects of overexpressing gK, like inhibition of viral glycoprotein synthesis and transport and accumulation of virus particles in the perinuclear space as a result of collapsing Golgi complex, are similar to the effects seen in the presence of the chemical inhibitor brefeldin A (Cheung et al., 1991).

Replication of herpesviruses involves synthesis of DNA, assembly of capsids which are filled with DNA, transportation of capsids through the nucleocytoplasmic barrier, formation of tegument and envelope, and transportation to the cell periphery for release into the extracellular space. gK deficient mutants produce no infectious progeny. The presence of naked, DNA filled capsids within the cytoplasmic matrix indicates that assembly of capsids, DNA filling and transportation of capsids trough the nucleocytoplasmic barrier is not disturbed. The questions arise whether tegumentation or formation of the viral envelope is disturbed or whether intact virions are produced that lack essential proteins to be infective. To address these questions, we constructed a recombinant gK-null mutant in the HSV-1 (F) genetic background. Characterization of replication and ultrastructural characteristics of these recombinant viruses revealed that the number of virus particles produced in the absence of gK was equal to that of HSV-1 infected cells. However, these virus particles failed to be enveloped indicating that gK plays an essential role in budding of capsids at cell membranes.

Materials and Methods

Cells and viruses. African green monkey (Vero) cells and HeLa GT-GFP cells stably expressing a fusion of galactosyltransferase (GT) membrane domain and green fluorescence protein (GFP) (Senn et al., 2007) were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM: Gibco, Bethesda, MD, USA) supplemented with 10% fetal calf serum (FCS; Gibco) in the presence of 5% CO_2. Wild-type virus strain HSV-1 (F) was propagated in Vero cells. For purification of virus particles, infected cells were lysed by sonication and the suspension was centrifuged at 1,000 x g for 10 min. The pellet was discarded and the supernatant was centrifuged through a 25% (w/v) sucrose cushion in Hank's Balanced Salt Solution (HBSS) at 100,000 x g for 3 h. The virus pellet was resuspended in HBSS buffer. Titers of virus stock were determined on Vero cell monolayers.

Construction of transfected cell line. A plasmid expressing HSV-1 UL53 with an hemagglutinin (HA) tag under the promoter of AAV p5 was constructed as follows. First, UL53 sequence was amplified by PCR from pYEbac102, harbouring full-length HSV-1 (F) genome (Tanaka et al., 2003), using a reverse primer containing nucleotides encoding an HA tag (primer forward, 5'-gat caa gct tac gcc ATG CTC GCC GTC CGT TA–3': primer reverse, 5'–gat ctc tag aTC Acc atg gag cat aat ctg gaa cat cat atg gat aTA CAT CAA ACA GGC GCC TCT GGA–3'; UL53 sequences are shown in capital letters). The PCR product was digested with HindIII and XbaI and inserted into the corresponding sites in the pcDNA3.1+ vector (Invitrogen, Basel, Switzerland) to generate pCS175. Next, the CMV promoter of pCS175 was replaced by the AAV p5 promoter. The p5 promoter of pBS-p5-tetO [AAV2 nt 152 to 299, (Glauser et al., 2005)] was excised with Ecl136I and BamHI and cloned into pCS175, which was digested with HindIII, blunted using Klenow, and digested with BglII. The resulted plasmid was named pCS197. A gK complementing cell line was obtained by transfecting Vero cells with SspI linearized pCS197 using Lipofectamine (Invitrogen). Two days after transfection, cells were seeded into medium containing 500 µg/ml G418. Single antibiotic-resistant cell colonies were isolated and tested by PCR for integration of UL53-HA gene and assayed for ability to complement gK deletion mutant. Based on number and size of plaques formed, one of these colonies (vCS197) was chosen for use in all subsequent studies.

Construction of recombinant viruses. The HSV-1 UL53 deletion mutant was obtained by homologous recombination in *E. coli* strain SW102 maintaining pYEbac102. UL53 sequence was replaced with a galK expression cassette, which was amplified by PCR using pgalK. Homology arms flanking UL53 were added to the primers (primer forward, 5'-tct tcg gtg cca gtc cgc tgc acc gat gta ttt acg cgg tac gcc cca ccg CCT GTT GAC AAT TAA TCA TCG GCA-3'; primer reverse, 5'-gtt tcc aat ttg cat atg ccg tta cgg ttt ccg ccg gcc tgg atg tga cgt TCA GCA CTG TCC TGC TCC TT-3'; galK sequences are shown in capital letters). The DpnI digested und purified DNA fragment was electroporated into competent *E. coli* strain SW102. Recombinant colonies were selected for growth on galactose plates (Warming et al., 2005). To generate gK deficient viruses, purified recombinant DNA of pYEbac102ΔUL53 was cotransfected with pCre (plasmid harbouring Cre recombinase under CMV promoter) into vCS197 cells. The resulting viruses were plaque-purified three times under an overlay medium containing 1% agarose. DNA of the resulted viruses were isolated and verified by PCR amplification, restriction enzyme fragment analysis and Southern blotting with a

digoxigenin-labeled probe. One gK deficient recombinant shown to lack the coding domain of gK as well as the BAC sequence was designated as CSΔgK (Fig. 1).

The rescue mutant was created by recombination between DNA sequence containing UL53-HA gene and pYEbac102ΔUL53 DNA. Plasmid pCS177 containing the UL53 gene with a carboxy terminal HA tag and flanking sequence to UL53 that extend 300 bp upstream and 400 bp downstream of the UL53 gene of HSV-1 was constructed in the following way. First, the downstream sequence of UL53 was amplified by PCR from HSV-1 (F) genome (primer forward, 5'-gat ctc tag acg tca cat cca ggc cgg cgg aa-3'; primer reverse, 5'-gat cga gct cag gCC TCC GGC ACA GAC AAG GAC CAA T-3'; HSV-1 sequences are shown in capital letters). The resulting PCR product was cut with SacI and XbaI and cloned into the SacI and XbaI sites in pBluescript II KS(+), resulting in plasmid pCS176. Second, the UL53 gene with its upstream flanking sequence was amplified by PCR from HSV-1 (F) using a reverse primer containing nucleotides of HA tag (primer forward, 5'-gat caa gct tag gcc tgg gtc ggt aca acg tac agc cgg at-3'; primer reverse, 5'-gat ctc tag aTC Acc atg gag cat aat ctg gaa cat cat atg gat aTA CAT CAA ACA GGC GCC TCT gga-3'; HSV-1 sequences are shown in capital letters). Following PCR, the DNA product was digested with HindIII and XbaI and inserted into these sites in pCS176, resulting in plasmid pCS177. The StuI fragment of pCS177 containing UL53-HA gene with flanking sequence was cotransfected together with pYEbac102ΔUL53 and pCre into Vero cells. Plaques were subjected to three cycles of plaque purification. gK-HA expression was identified using indirect immunofluorescence. One virus stock expressing gK-HA was designated as rCSgK. The genomic structure of the rescue mutant was confirmed by Southern blot analysis.

Antibodies. The anti-HA monoclonal antibody was obtained from Santa Cruz Biotechnology. The polyclonal antiserum raised against the HSV-1 tegument protein VP22 was kindly provided by Dr. Bernhard Roizman, University of Chicago, USA. MAb 3114 specific for HSV-1 gE was a gift from Nigel Stow, Institute of Virology, Glasgow, United Kingdom. Anti-ICP4 was purchased from Advanced Biotechnologies Inc, Columbia, Maryland, USA. Goat anti-mouse antibodies conjugated to Alexa-594 was obtained from Molecular Probes, Basel, Switzerland and goat anti-rabbit antibodies conjucated to fluorescein (FITC) was obtained from Southern Biotechnology Associates Inc., Birmingham, USA.

Indirect immunofluorescence. Cells were grown on glass coverslips for 24 h and infected with the indicated virus. At the stated times cells were washed with phosphate-buffered saline (PBS), fixed with 3% paraformaldehyde at room temperature for 20 min and quenches with 50 mM glycine. After cells were made permeable by immersion in 0.02% Triton-X 100 for 5 min, cells were blocked by incubation with 2% bovine serum albumin in PBS. Antibodies diluted in blocking solution were added to the samples and incubated at room temperature for 60 min. Cell nuclei were stained with 1 µg/ml DAPI (Roche, Reinach, Switzerland) for 5 min. samples were embedded in fluorescence mounting medium (DakoCytomation, Glostrup, Denmark) and examined using a standard or confocal laser scanning microscope (SP1, Leica, Mannheim, Germany). Images from confocal microscopy were deconvolved with a blind deconvolution algorithm using the Huygens Essential program suite (SVI, Hilversum, The Netherlands) and processed with Imaris 4.1.1 (Bitplane AG, Zurich, Switzerland).

Complementation assay. Complementation of the gK deficient HSV-1 with either BoHV-1 gK or HSV-1 gK was carried out in Vero cells. BoHV-1 gK was amplified by PCR using f115 (Tobler et al., 2002) as template (primer forward, 5'-gat caa gct tcc acc ATG CTG CTC GGG GGG CGG ACT-3'; primer reverse, 5'-acg tgg atc cTCA agc ata atc tgg aac atc ata tgg ata CGT CTG CGC GCC CAG CA-3'; BoHV-1 sequences are shown in capital letters). The resulting PCR product was inserted into pHSVPrPUC (Bergold et al., 1993), obtaining plasmid pCS166. pCS174 was constructed in the same way as pCS166, except that HSV-1 gK fragment was used instead of BoHV-1 gK fragment. HSV-1 gK was amplified from pYEbac102 by PCR (primer forward, 5'-gat caa gct tac gcc ATG CTC GCC GTC CGT T-3'; primer reverse, 5'-gat ctc tag aTC Acc atg gag cat aat ctg gaa cat cat atg gat aTA CAT CAA ACA GGC GCC TCT GGA-3'; HSV-1 sequences are shown in capital letters). Both plasmids pCS166 and pCS174 contain BoHV-1 gK and HSV-1 gK coding sequence, respectively, under control of the HSV-1 IE4/5 promoter. pYEbac102 DNA was transfected either alone or together with either plasmid pCS166 or pCS174 into Vero cells using Lipofectamine (Invitrogen). 72 h pi, cells were fixed and indirect immunofluorescence studies were performed.

Low-temperature transmission electron microscopy. Cells grown on sapphire disks were infected with an MOI of 5 and were frozen at indicated time in a high-pressure freezer (HPM010; BAL-TEC Inc., Balzer, Liechtenstein). The frozen cells were freeze-substituted with acetone containing 0.25% glutaraldehyde and 0.5% osmium tetroxide and embedded in

Epon (Wild et al., 2002). Ultrathin sections were analyzed in a transmission electron microscope (CM12, Philips, Eindhoven, the Netherlands) equipped with a slow scan charge coupled device camera (Gatan, Pleasanton, CA).

Morphometric analysis. Surface area as well as the volume of the Golgi complex was estimated by morphometric analysis on randomly selected cells at a final magnification of 89'500 applying the point counting method with a multipurpose test system (Weibel, 1979). Intersection (Ig) of test lines with Golgi membrane and points hitting the cytoplasm (Pcy) were determined to calculate the surface density of the Golgi complex (Svg) on the basis of the equation Svg=Ig/Pcy*d, whereby d is the test line length. The volume density (Vvg) was calculated using the formula Vvg=Pg/Pcy, whereby Pg are points hitting the lumen of Golgi complex. The data are expressed as μm^2 surface area or as μm^3 volume per 1000 μm^3 cytoplasm.

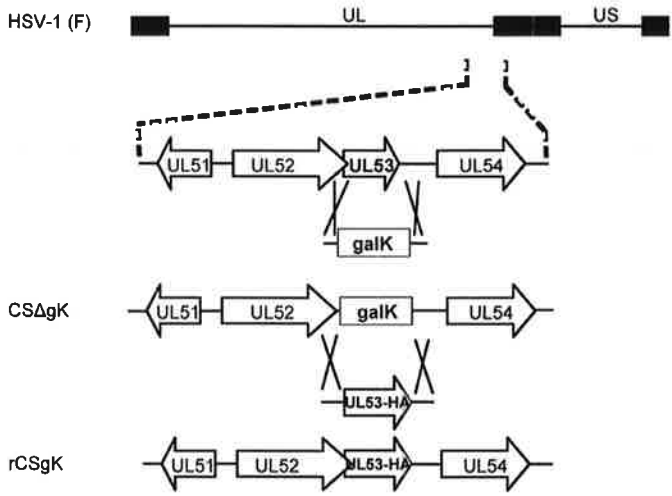

Fig. 1. **DNA sequence arrangements in the genome of wild-type HSV-1, CSΔgK and rCSgK.** The top line represents the arrangement of the wild-type HSV-1 (F) genome with the unique long (UL) and unique short (US) regions flanked by the inverted repeats. Depicted below is the region of HSV-1 DNA containing open reading frames UL51, UL52, UL53 and UL54. Arrows indicate transcriptional orientations. CSΔgK was generated by replacing the open reading frame of UL53 with a galK cassette. rCSgK was derived from CSΔgK by substituting the galK cassette with a cloned fragment harbouring HSV-1 gK containing an HA tag and flanking sequence to UL53.

Results

Construction of cell line expressing gK protein under p5 promoter. It was demonstrated that gK is essential for virus replication in cultured cells. Therefore, a complementing cell line was required to allow replication of gK deficient mutant. To avoid the potential toxicity of gK, we constructed a cell line expressing gK from the inducible AAV p5 promoter that is only active upon infection with herpesvirus or adenovirus. We constructed a plasmid in which the AAV2 p5 promoter region was cloned upstream of UL53-HA coding sequence. The UL53 open reading frame was tagged with an HA tag just in front of the stop codon. Vero cells were transfected with the resulting plasmid encoding UL53-HA and a neomycin resistance gene. Following selection with antibiotics, resistant colonies began to appear after 10-15 days on plates containing transfected cells. In contrast, no resistant colonies were observed in untransfected Vero cells. The clone vCS197 was selected from neomycin-resistant clones based on the integration of HA tagged-UL53 gene. Single clones of the transformants were maintained and were tested for the integration of HA-tagged UL53 gene by PCR and for the competency to complement gK deficient mutant (Fig. 4D).

The controlled expression and cellular localisation of gK in vCS197 cells were studied by confocal microscopy. Vero and vCS197 cells were either mock infected or infected with HSV-1 followed by fixation and staining with antibodies against HA (Fig. 2). HSV-1 infection activates the p5 promoter in vCS197 and gK-HA protein was visible as bullet-like shapes localized in the perinuclear region 24 h pi. Anti-HA antibodies gave neither a signal in mock infected vCS197 cells nor in HSV-1 infected Vero cells. These findings indicate that UL53 expression in vCS197 cells is dependent on HSV-1 infection and confirmed that p5 promoter is silent in the absence of virus infection. The inducibility of gK expression remained stable after many cell passages. The stability of vCS197 cells for the HSV-dependent activation of UL53 was confirmed by repetition of the immunofluorescence assay after culturing for more than one month.

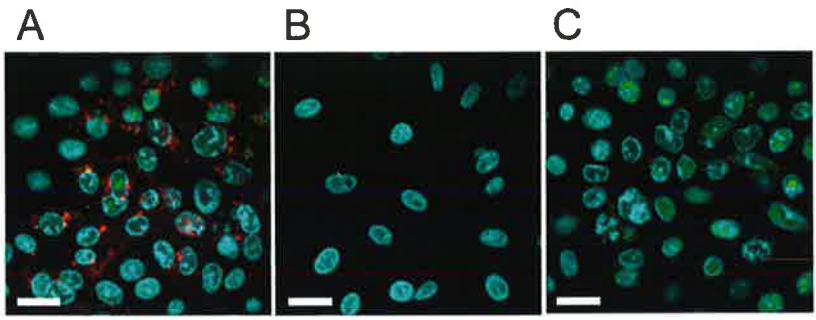

Fig. 2. **Immunofluorescence staining of the stable cell line vCS197 expressing HSV-1 gK-HA.** vCS197 were infected with wild-type HSV-1 (A) or were mock infected (B). Vero cells were infected with wild-type HSV-1 (C). Cells were fixed 24 h pi, permeabilized and double stained with a polyclonal antibody against HSV-1 VP22 (green) and with a monoclonal antibody against HA (red). Cell nuclei were stained with DAPI (blue). Bars, 20 µm.

Recombinant viruses construction. For investigating the significance of gK for HSV-1 life cycle, a UL53 deletion mutant (CSΔgK) was generated by mutagenesis of the HSV-1 BAC clone YEbac102 in *E. coli* strain SW102. To avoid any disrupting expression of the neighbouring genes, only the C-terminal 312 amino acids of the total 338 amino acids of UL53 were deleted (Fig. 1). The UL52 gene terminates within the UL53 coding sequence and is essential for viral replication. The polyadenylation signal of UL52 was not disrupted by deleting UL53 gene because it is located downstream of the UL53 gene. After cotransfection of YEbac102ΔUL53 together with a plasmid expressing Cre recombinase into Vero cells or vCS197 cells, isolation of infectious CSΔgK virus was only successful on vCS197 cells. Resulting viruses were plaque purified on vCS197 cells. Proper insertion of the recombination fragment within the HSV-1 genome was confirmed by restriction enzyme analysis and Southern blot analysis. In the revertant mutant, rCSgK, the deletion of UL53 was repaired. rCSgK was constructed by cotransfection of YEbac102ΔUL53, a plasmid harbouring Cre recombinase and with a DNA fragment containing UL53-HA tagged sequence with UL53 flanking sequences. For discrimination between revertant mutant and wild-type parental HSV-1, UL53 was tagged at its carboxyterminus with an HA epitope in front of the stop codon in the revertant mutant. Progeny viruses were subjected to three additional rounds of plaque purification. Viral genomic DNA was purified from the infected cell lysates, and correct recombination was confirmed by PCR and immunofluorescence (Fig. 3).

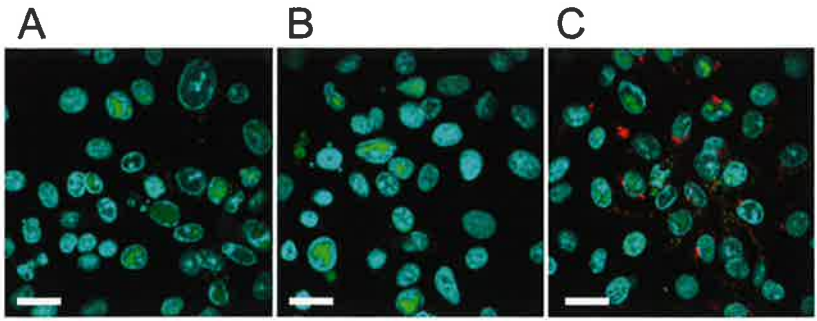

Fig. 3. **Immunofluorescence staining of infected Vero cells.** Cells were infected with wild-type HSV-1 (A), CSΔgK (B) or rCSgK (C). 36 h pi, infected cells were fixed and prepared for indirect immunofluorescence using polyclonal antibodies against HSV-1 VP22 (green) and monoclonal antibodies against HA tag (red). Cell nuclei were stained with DAPI (blue). Bars, 20 µm.

Plaque formation was obstructed in the absence of gK. In plaque assays, only single infected cells or small foci of infected cells were observed in Vero cells infected with CSΔgK (Fig. 4). Plaque formation was even not observed after one week post infection, confirming that HSV-1 gK is essential for virus replication in cell culture. Plaque formation could be restored either with the revertant mutant rCSgK or on gK expressing cell line vCS197. Plaque formation in CSΔgK infected vCS197 cells was as efficient as in parental HSV-1 infected Vero cells. Since vCS197 cells carry and express only the coding sequence of UL53 and no other viral proteins, but are competent to rescue the defects of the mutant virus, the observed defects are most likely due to the introduced gK deletion and not to other fortuitous alterations elsewhere in the genome. It is worth mentioning that even after many virus passages in vCS197 no recombination could be observed between complementing cell line vCS197 and viral genome CSΔgK.

BoHV-1 gK provided in *trans* cannot complement HSV-1 gK. BoHV-1 gK and HSV-1 gK are essential for production of infectious virus particles. HSV-1 gK and BoHV-1 gK are 53% and 34% identical on the DNA and amino acids levels, respectively. The conservation of primary structure of HSV-1 gK and BoHV-1 gK as well as their contingent related functions prompted us to analyse whether expression of BoHV-1 gK can compensate for the lack of HSV-1 gK in viral genome by restoring viral replication. pYEbac102ΔUL53 was cotransfected with plasmid either expressing BoHV-1 gK (pCS166) or expressing HSV-1 gK

(pCS175). 72 h pi, plaques were visible in monolayers transfected with pYEbac102ΔUL53 and HSV-1 gK encoding plasmid. However, only single cells or small foci were detected in monolayers transfected either with pYEbac102ΔUL53 alone or together with plasmid expressing BoHV-1 gK (Fig. 5). Even after 1.5 week plaques could not been observed in cells transfected with pYEbac102ΔUL53 together with pCS166, suggesting that BoHV-1 gK may not compensate for the gK deletion of HSV-1. It should be noted that the functionality of BoHV-1 gK in Vero cells was not evaluated.

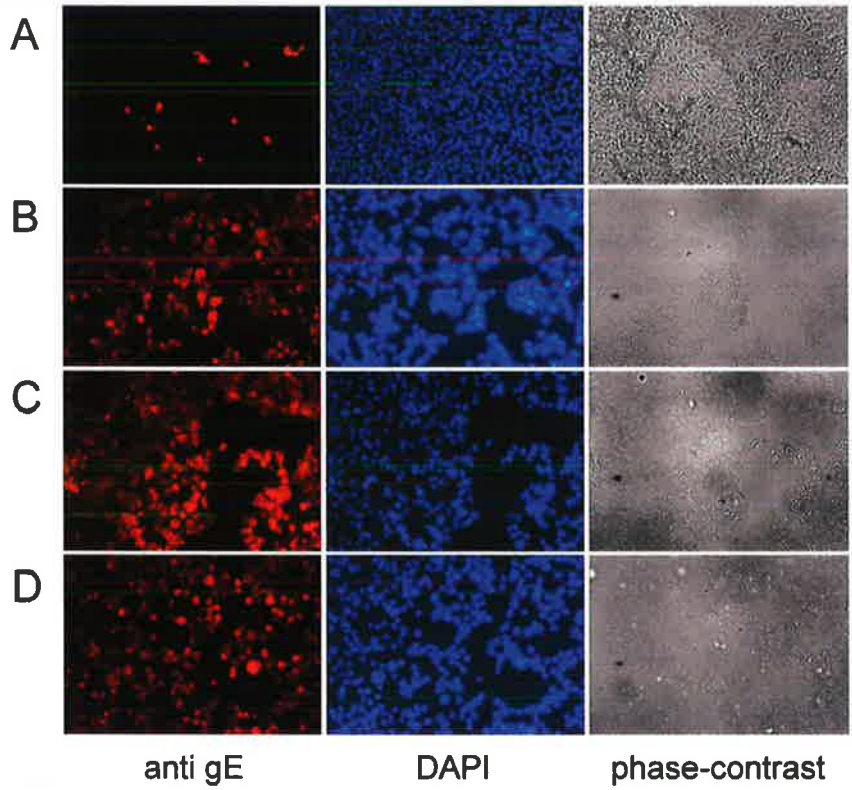

Fig. 4. **Plaque formation of wild-type and virus mutants.** Monolayers of Vero cells were infected either with CSΔgK (A), with wild-type HSV-1 (B) or with rCSgK (C). Plaque formation of CSΔgK could be restored by infection of vCS197 with CSΔgK (D). 48 h pi, cells were fixed and prepared for indirect immunofluorescence. Cells were incubated with antibodies against HSV-1 gE for the detection of infected cells. Cell nuclei were stained with DAPI.

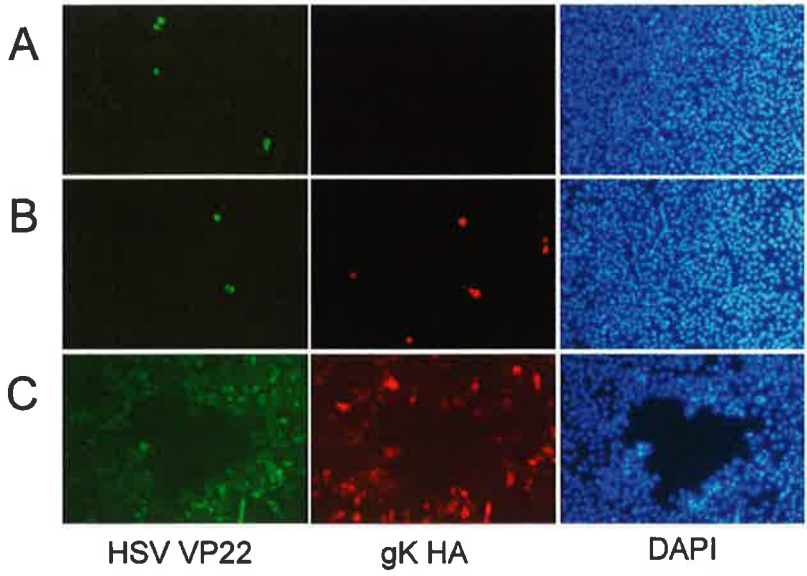

Fig. 5. **Plaque formation after cotransfection of Vero cells.** Vero cells were cotransfected with pYEbac102ΔUL53 and either (A) with an empty plasmid, (B) with a plasmid expressing BoHV-1 gK (pCS166), or (C) with a plasmid expressing HSV-1 gK (pCS174). After 72 h, cells were fixed and prepared for immunofluorescence microscopy using antibodies directed against HSV-1 protein VP22 for detection of infected cells and antibodies against HA tag. Both HSV-1 gK and BoHV-1 gK were tagged with an HA epitope. Nuclei were stained with DAPI.

The number of enveloped virus particles is drastically reduced in the absence of gK. Electron microscopy was employed to identify intracellular localization of CSΔgK virus and wild-type HSV-1 in Vero cells. The total virus particles counted in 20 randomly selected cell sections were similar in Vero cells infected with HSV-1 compared to CSΔgK infected Vero cells at given times. However, the distribution of capsids and enveloped virions within the cytoplasm differed dramatically (Fig. 6). In HSV-1 infected cells up to 65% of total counted virus particles were enveloped, whereas only 3.2% of counted virus particles were enveloped in CSΔgK infected cells. The number of virions within perinuclear space, rough endoplasmic reticulum and Golgi was significantly decreased in CSΔgK infected cells compared to HSV-1 infected cells at all indicated times. In the absence of gK, 98.7% of total counted CSΔgK virus particles were naked capsid in the cytoplasm at 20 h pi, while significantly fewer

capsids (33%) were counted in the cytoplasmic matrix in HSV-1 infected cells. The cytoplamic capsids in CSΔgK infected cells were dispersed throughout the cytoplasm and did not form aggregates. Although cytoplasmic capsids interact with the inner nuclear membrane as well as with Golgi membrane, it seems that the budding process cannot be completed in the absence of gK (Fig. 7). The low number of enveloped particles in CSΔgK infected cells indicates a major defect in virus egress. These defects were not seen by examining vCS197 cell line infected with CSΔgK. The revertant mutant rCSgK also showed normal virus maturation in Vero cells as HSV-1.

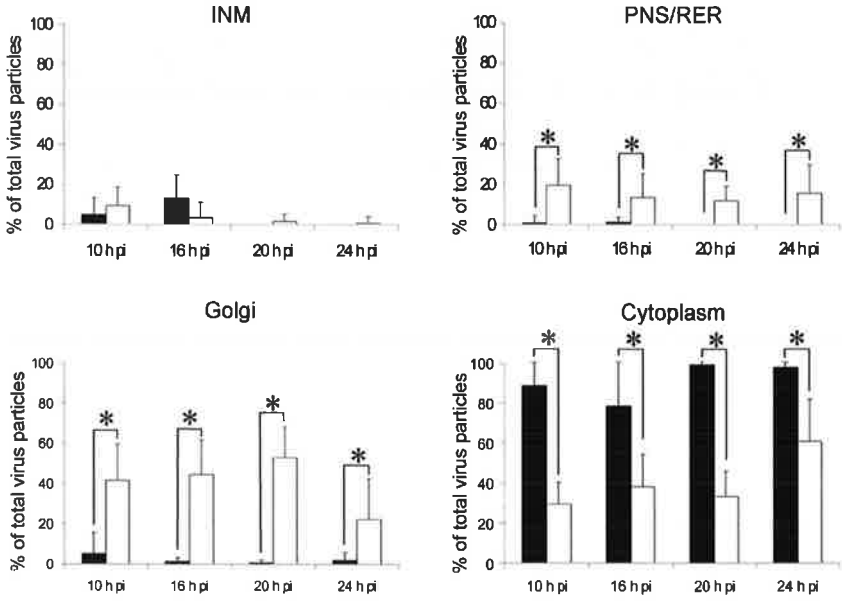

Fig. 6. **Percentages of virus particles in cellular compartments.** Distribution of virus particles presented in infected Vero cells were determined at times indicated. Virus particles were counted in electron micrographs of 20 randomly selected cells infected with CSΔgK (black bar) and wild-type HSV-1 (white bar). The average number of total counted virus particles found in infected cells was defined as 100%. Bars show the average number of capsids interacting with the inner nuclear membrane (INM), enveloped virions within the PNS and RER (PNS/RER), enveloped virions within Golgi cisternae and Golgi vesicles (Golgi) or naked capsids in the cytoplasmic matrix (Cytoplasm). Mean and standard deviations are shown. Data were compared by a multiple *t*-test (*$p<0.001$).

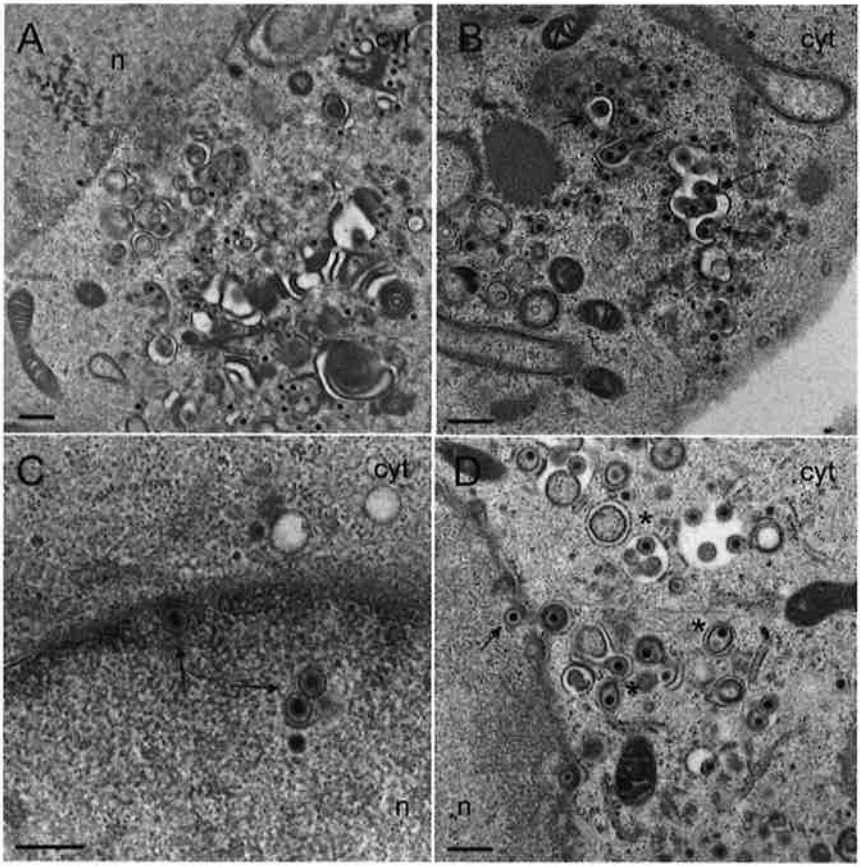

Fig. 7. **Ultrastructural morphology of CSΔgK and rCSgK viruses 24 h pi.** (A and B) Vero cells were infected with CSΔgK at an MOI of 5, incubated for 24 h and prepared for transmission electron microscopy. The majority of capsids were distributed in the cytoplasm (cyt) of CSΔgK infected cells. Fully enveloped virus particles within the perinuclear space or within Golgi membranes were absent. (B) Unfinished budding events at Golgi membranes were observed in CSΔgK infected cells (arrow). (C) vCS197 cells infected with CSΔgK and (D) Vero cells infected with rCSgK showed all stages of virion morphogenesis, including enveloped virus particles within the perinuclear space (arrows) and within Golgi derived vesicles (asterisk). n, nucleus. Bars, 500 nm.

Integrity of nuclear envelope is lost during virus replication. The nuclear surfaces of infected cells were examined in order to clarify how capsids gain access to the cytoplasm in the absence of gK. Electron microscopy revealed some capsids at early stages of budding at the inner nuclear membrane as indicated by thickening of the inner nuclear membrane that is

pushed into the perinuclear space (Fig. 8A). However, successfully completed budding events at the inner nuclear membrane were almost absent in CSΔgK infected cells compared to wild-type infected cells (Fig. 6).

Electron microscopy revealed massive gaps in the nuclear membrane of infected cells (Fig. 8B). Some of these gaps were distinctly bordered by the inner nuclear membrane continuing into the outer nuclear membrane and thus were assumed to represent impaired nuclear pores lacking nuclear pore complex structures. Within these nuclear membrane gaps, nuclear material protruded into the cytoplasm. Capsids were found in association with these considered impaired nuclear pores, indicating that they may escape through them into the cytoplasmic matrix.

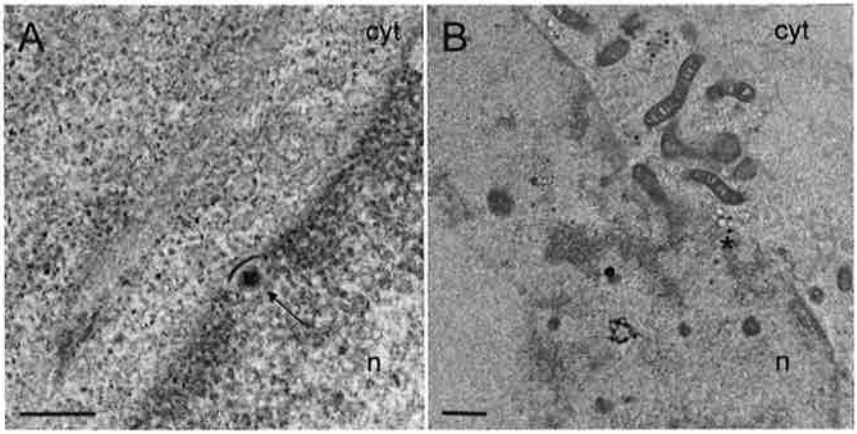

Fig. 8. **Ultrastructural examination of nuclear envelope of CSΔgK infected cells.** Vero cells were infected with CSΔgK at an MOI of 5. Cells were fixed at 24 h pi and processed for electron microscopy. (A) Capsid (arrow) at the beginning of budding at the inner nuclear membrane, which is thickened at the site of budding. In the absence of gK, enveloped virus particles within the perinuclear space were quite rarely. (B) Integrity of the nuclear membrane is lost during virus replication. Capsids were observed within nuclear membrane gaps (asterisk). n, nucleus; cyt, cytoplasm. Bars, 500 nm.

Golgi complex is intact in CSΔgK infected cells. Based on the finding that in the absence of gK almost no enveloped particles exist, Golgi fields were examined for their integrity in CSΔgK infected cells. At various times of incubation, the surface area and the volume of Golgi complex was determined by morphometric analysis in 30 randomly selected cells (Fig.

9). Cells were mock infected or infected either with CSΔgK viruses or wild-type HSV-1. In the course of infection from 9 h pi up to 24 h pi, no significant differences could be observed in the surface area of Golgi complex between CSΔgK infected cells, HSV-1 infected cells and mock infected cells.

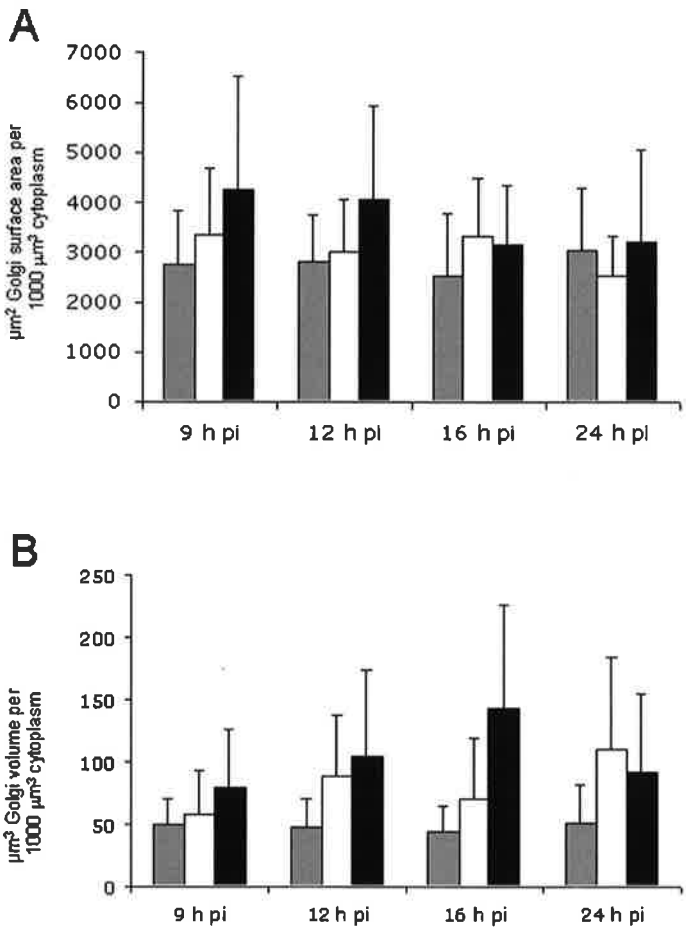

Fig. 9. **Quantitative analysis of Golgi size.** Cells were infected either with mock (grey bar), wild-type HSV-1 (white bar) or CSΔgK (black bar) at MOI of 5. Cells were frozen at times indicated and prepared for electron microscopy. 30 randomly selected cells were photographed and surface area (A) and volume (B) of the Golgi complex were morphometrically analyzed applying the point counting method. Mean and standard deviation of the results are shown.

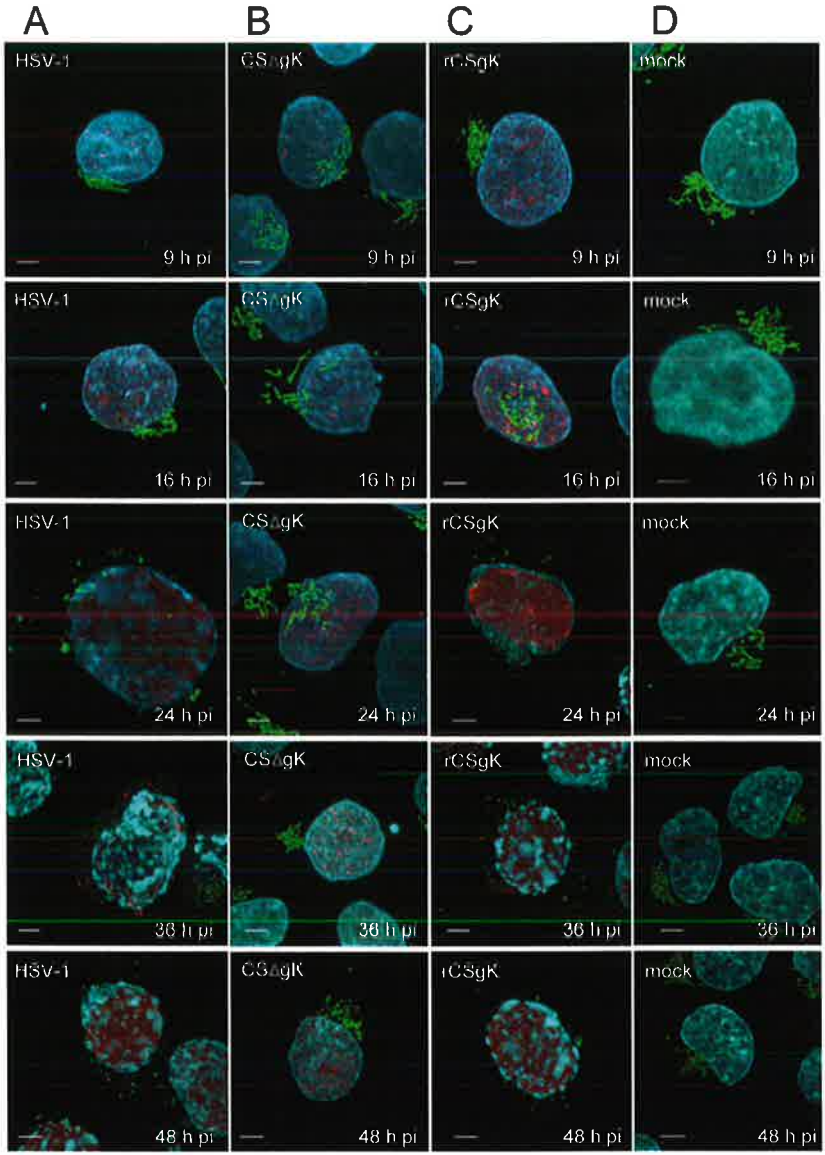

Fig. 10. **Three-dimensional distribution of *trans*-Golgi membranes (green; GT-GFP).** HeLa GT-GFP cells were infected either with wild-type HSV-1 (A), CSΔgK (B), rCSgK (C) or mock (D) for 9, 16, 24, 36 or 48 h and were analysed by confocal microscopy. Infection of cells was ascertained by immunolabeling the viral protein ICP4 (red) and nuclei were stained with DAPI (blue). Bars, 5 µm.

To reevaluate the findings of the morphometric analysis, confocal microscopy was performed of HeLa GT-GFP cells. Virus infected or mock infected HeLa GT-GFP cells were fixed after different times and stained with antibodies against ICP4 to ascertain infection (Fig. 10). Up to 16 h pi, Golgi apparatus was found in a typical ribbon-like shape localized in a perinuclear region in infected and mock infected examined cells. 24 h after infection, the structure of Golgi complex was altered in HSV-1 infected cells and in rCSgK infected cells. Only few GT-GFP spots were visible in the cytoplasm of HSV-1 and rCSgK infected cells, whereas in CSΔgK infected cells and also in mock infected cells Golgi complex showed still a tubular structure. Moreover, the Golgi complex seemed to be unaffected in CSΔgK infected cells even after 48 h pi, whereas in wild-type and revertant mutant infected cells Golgi complex was entirely fragmented.

Discussion

Since gK is essential for viral replication production of gK deficient mutants requires trans-complementing cell lines that provide the missing structural protein. Currently, complementing cell lines are widely utilized to obtain infectious virus particles of deficient viral genome *in vitro*. While constitutive promoters are well suited for use of expression of nontoxic proteins, constitutive expression of significant levels of HSV-1 gK is cytotoxic (Hutchinson et al., 1992). Overexpression of gK was shown to result in disrupting of the Golgi apparatus (Foster et al., 2003b). Therefore, complementing cell lines with the authentic promoter of HSV-1 gK would give adequate timing and expression levels of gK. However, unwanted recombination between cellular genome and viral genome can occur with adding additional HSV-1 sequences (Cai et al., 1987; Roop et al., 1993). To overcome these difficulties, we generated vCS197, a stable cell line which expresses HSV-1 gK from the AAV p5 promoter. AAV requires co-infection with a helper virus, such as adenovirus or herpesvirus, for productive infection (Berns and Linden, 1995). In the presence of helper virus co-infection, the synthesis of all AAV proteins is readily induced. When HSV-1 serves as helpervirus, the ICP0 transactivator protein of HSV-1 induces transcription from the p5 promoter (Geoffroy et al., 2004). Indeed, infection of vCS197 cells with HSV-1 virus induces gK expression from the AAV p5 promoter, whereas no gK expression was visible in mock infected vCS197 cells (Fig. 2).

Deletion of the UL53 gene in the HSV-1 genome resulted in defects of plaque formation. Since the UL52 and UL53 open reading frames partly overlap only the C-terminal 312 amino acids of the total 338 amino acids of UL53 were deleted without disturbing the coding sequence of UL52. UL52 is unlikely to be responsible for the phenotypes observed by CSΔgK. The UL52 protein is essential for synthesizing viral DNA (Klinedinst and Challberg, 1994). Thus, any substantial defect in UL52 function should be reflected in failure to produce any DNA-containing capsids. Cells infected with CSΔgK, however, produce DNA-containing capsids suggesting that the defects observed in CSΔgK infected cells were not due to an insufficiency of UL52 protein. The observed defects of CSΔgK can be obviated either by complementation of UL53 in *trans* or by homologues repair of the UL53 locus (Figs. 4C, 4D, and 5C). There is no evidence for a specific failure to express other viral proteins. Therefore, the observed phenotypes from CSΔgK are solely due to the lack of UL53 sequence.

It is worth mentioning that gK is conserved in all alphaherpesviruses suggesting that there may be conservation of function of this glycoprotein in some common aspect of the virus life cycle (Foster and Kousoulas, 1999; Mo et al., 1999).

Both viruses, HSV-1 and BoHV-1, belong to the subfamily alphaherpesvirinae and can replicate in Vero cells (Ono et al., 2004). *In vitro* growth properties of gK-negative HSV-1 and gK-negative BoHV-1 are similar, both the BoHV-1 and HSV-1 gK protein are essential for viral replication in cell culture (Hutchinson and Johnson, 1995; Senn). Although gK of BoHV-1 contains putative glycosylation sites and hydrophobic domains, which are similar in structure and position to those of gK HSV-1, gK of BoHV-1 did not compensate for the lack of HSV-1 gK by restoring viral replication in this study (Fig. 5). Interestingly, Mo et al. (Mo et al., 1999) demonstrated that also HSV-1 gK could not compensate for the deletion of VZV gK. These observations suggest that the function of these gK homologues may not be identical in heterologues herpesviruses although gK deficient mutants show the same phenotype. Furthermore, cellular localisation of homologues gK may differ in cells, or interaction of gK with other viral factors, which may be required for efficient virus replication, only occurs when the appropriate homologues viral factors are present.

Our analysis of gK deletion mutant demonstrated that HSV-1 gK is critical for viral egress confirming that gK is an essential protein for virus replication like its homologous in EHV-1, VZV and PRV (Klupp et al., 1998; Mo et al., 1999; Neubauer and Osterrieder, 2004). CSΔgK produced virtually no infectious virus particles prompting us to search for defects in the

egress pathway using electron microscopy. The results of this analysis should be seen in the context of the three prevailing models postulated for HSV-1 egress. One model, the vesicle formation model, postulates that nucleocapsids acquire their final envelope by budding at the inner nuclear membrane and escape from there via vesicle formation. These vesicles then pass the Golgi complex for final maturation of virions (Torrisi et al., 1992). In a second model, the deenvelopment model, it is hypothesized that the viral envelope derived from budding at the inner nuclear membrane fuses with the outer nuclear membrane releasing capsid into the cytoplasm. Capsids acquire the second envelop by budding at membranes of the *trans*-Golgi network (Skepper et al., 2001). The third model suggests a dual pathway for egress (Wild et al., 2005). Capsids gain access to the cytoplasmic matrix via impaired nuclear pores. For acquiring envelope and tegument, they bud at membranes of rough endoplasmic reticulum or the Golgi complex. Alternatively, virions derived by budding at the inner nuclear membrane are intraluminally transported via rough endoplasmic reticulum and into Golgi cisternae (Leuzinger et al., 2005). Quantitative ultrastructural analysis of CSΔgK infected cells displayed up to 98% of the total count of virus particles to be naked capsids located within the cytoplasmic matrix. This observation can be reconciled with either of the two above first mentioned current models for HSV egress. In the vesicle formation model such cytoplasmic capsids are interpreted as dead-end products resulting from inappropriate fusion of the virion envelope with the outer nuclear membrane (Campadelli-Fiume et al., 1991). In contrast, cytoplasmic capsids are viewed as functional intermediates in the deenvelopment model (Smith, 1980). In both models, the observed cytoplasmic capsids require primarily budding at the inner nuclear membrane. In CSΔgK infected cells less than 2% of capsids were found within the perinuclear space at all examined times (Fig. 6). Therefore, fusion of the viral envelope with the outer nuclear membrane, which would lead to this high number (98%) of cytoplasmic capsids in the absence of gK, is unlikely. Consequently, capsids need to exit the nucleus through another way. Scrutinizing of the nuclear membrane of CSΔgK infected cells revealed gaps in the nuclear envelope 24 h pi (Fig. 8B). Nuclear material protruded through such gaps into the cytoplasm suggesting that capsids can exit the nucleus via these nuclear envelope gaps. It was proposed in the past for BoHV-1 and HSV-1 that capsids can leave the nucleus via impaired nuclear pores (Leuzinger et al., 2005; Wild et al., 2005). In conclusion, the observed phenotype of CSΔgK is best understood with the dual pathway model. All the capsids found in the cytoplasm are proposed to leave the nucleus via observed nuclear membrane gaps.

The formation of infectious HSV-1 virions involves budding of capsids through a cellular membrane, acquisition of tegument proteins, and release of intravesicular enveloped virus at the cell surface. The molecular details of how HSV-1 becomes enveloped at either nuclear or cytoplasmic membranes are poorly characterized. In the case of cytoplasmic envelopment, it is evident that viral membrane glycoproteins accumulate extensively in the *trans*-Golgi network and this likely promotes incorporation into the virion envelope (Farnsworth and Johnson, 2006; Turcotte et al., 2005). The cytosolic domains of these membrane glycoproteins probably provide a surface on which the last stage of assembly, i.e., acquisition of the virion envelope occurs. HSV-1 mutants lacking individual glycoproteins known to be essential for virus replication, gD, gB, gH, and gL, display no obvious defect in envelopment. In each case, normal numbers of enveloped particles are produced, although these particles are not infectious because they lack the machinery to enter cells (Browne et al., 2001; Pertel et al., 2001; Turner et al., 1998). In contrast, many other proteins of HSV-1 have been shown to be crucial for envelopment but are not strictly required (Baines and Roizman, 1992; Baines et al., 1991; Browne et al., 2004; Duffy et al., 2006; Reynolds et al., 2002). To date, only three tegument proteins, UL48, UL37 and UL36, and one membrane protein, gK, have been shown to be essential for HSV-1 envelopment (Desai et al., 2001; Desai, 2000; Foster and Kousoulas, 1999; Mossman et al., 2000). Viruses deficient in one of these essential proteins are unable to become enveloped. VP16 protein is encoded by UL48 gene and serves multiple functions during HSV-1 maturation. It is a strong transactivator of viral genes and it is also suggested that it is required for assembly and integration of an intact tegument (Elliott et al., 1995; Hancock et al., 2006; Smibert et al., 1994; Vittone et al., 2005). In the absence of either UL36 or UL37, clusters of unenveloped capsids were present in the nucleus and in the cytoplasm (Desai et al., 2001; Desai, 2000). These mutants were unable to acquire a tegument and an envelope structure which consequently leads to an abrogation of infectious virus production. Capsids of UL36 or UL37 deficient mutant were never bound to membranes, therefore, UL36 and UL37 are proposed to play an important role for tethering of tegument-coated capsids onto the envelope. However, the intracellular phenotype of CSΔgK appears to be different to the phenotype observed in UL36 or UL37 deficient mutant. CSΔgK capsids were often observed in close contact to the nuclear envelope and adjacent to Golgi membranes. Therefore, the transport of capsids to budding sites was intact in the absence of gK. But, it seems that envelopment process *per se* is impaired in CSΔgK infected cells.

The most striking phenotype of CSΔgK was the absence of infectious virus particles. This absence of infectious virus particles was due to the lack of envelopment. In the absence of gK, less than 4% of capsids were enveloped, whereas the majority of wild-type capsids are enveloped (up to 66%; Fig. 6). Capsids budding at cellular organelles require large amounts of membranes upon progress of viral multiplication. In wild-type infected cells, Golgi complex was fragmented 24 h pi, implying that membranes of the Golgi complex were depleted for envelopment of cytoplasmic capsids and for transportation of virus particles to the extracellular matrix (Avitabile et al., 1995; Campadelli et al., 1993). Interestingly, Golgi complex in CSΔgK infected cells was found to be intact even after 48 h pi (Fig. 10). This observation indicates that Golgi membranes were not consumed in CSΔgK infected cells. In addition, also the surface area of Golgi complex seemed to be similar in CSΔgK infected cells compared to mock and HSV-1 infected cells (Fig. 9). Furthermore, Jayachandra et al. shows that Golgi complex is functional in the absence of gK (Jayachandra et al., 1997). The group demonstrated that essential glycoproteins, such as gB, gD and gH, were localised in the plasma membrane after infection with a gK deficient mutant. Therefore, lack of envelopment in the absence of gK is not due to an impaired Golgi complex.

In conclusion, we demonstrated that HSV-1 gK is required for envelopment at the inner nuclear membrane as well as at Golgi membranes. Virus particles were found interacting with cellular membranes but successful envelopment was blocked in the absence of gK. Therefore, formation of infectious virus particles was deficient in CSΔgK infected cells. In addition, this study has contributed to shed light into the herpesvirus egress quarrel. Our data are most consistent with the notion that impaired nuclear pores are the main route for capsids into the cytoplasm. Therefore, our data supports the dual-pathway model for HSV-1 egress. Further studies are required to understand the function of gK in respect of regulation of membrane fusion.

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Chapter 4

Glycoprotein G is Essential for Golgi Dependent Intracellular Herpes Simplex Virus 1 Transportation

Abstract

The Golgi complex is important for the life cycle of cells and of enveloped viruses. Although seemingly essential for envelopment and exit of Herpes simplex virus 1 (HSV-1), Golgi-breakdown is observed at late times during HSV-1 replication. We suspected that glycoprotein G (gG) of HSV-1, a viral envelope protein without denominated function, may affect this process. Using a gG deficient HSV-1 (CSΔUS4) and a revertant mutant, we observed that CSΔUS4 caused a significant, premature reduction of volume and surface area of the Golgi complex in Vero cells. Disassembly of Golgi complex was also visualized in CSΔUS4 infected cells expressing a fluorescent Golgi marker. In the absence of gG, virions accumulated within the perinuclear space and the rough endoplasmic reticulum. Reduced envelopment of CSΔUS4 virions by Golgi membranes and inefficient intraluminal transportation of viral particles to the cell periphery were a consequence. Thus, gG sustains the integrity of the Golgi complex during HSV-1 infection and protects its transportation capacity.

Introduction

The Golgi complex plays a key role in secretory cells including modification of secretory products and making membranes available for packaging secretory products into membrane bound entities that carry the content to the cell periphery for exocytotic release (Bennett, 1984). Enveloped viruses make use of the membrane bound function of the Golgi complex. Herpesviruses depend to a large extend on the functionality of the Golgi complex. Therefore, they can be used to address questions concerning Golgi functions.

Herpes simplex virus 1 (HSV-1), a complex viral structure, is composed of capsid, tegument and lipid bilayer envelope (Rixon, 1993). The viral envelope is obtained by budding at nuclear membranes or at Golgi membranes during the egress pathway (Leuzinger et al., 2005). The obtained viral envelope is studded with viral glycoproteins. Some of the glycoproteins are essential for attachment of virions to and entry into host cells. Other glycoproteins are nonessential for virus replication but assist in the course of infection (Roizman, 2001). However, even if nonessential glycoproteins appear to be dispensable during replication of virus, they presumably contribute to important aspects in the virus life cycle.

Glycoprotein G (gG) is one of the enveloped glycoproteins shown to be dispensable for virion infectivity and the function of it has been resistant to detailed characterisation. gG homologues have been identified in the majority of alphaherpesviruses (Bryant et al., 2003, Costes et al., 2006, Weber et al., 1987). In some viruses, such as bovine herpesvirus 1 (BoHV-1), equine herpesvirus 1, herpes simplex virus 2, infectious laryngotracheitis virus, and pseudorabies virus (PRV), gG is proteolytically processed and secreted into the medium of infected cells (Crabb et al., 1992; Drummer et al., 1998; Engelhardt and Keil, 1996; Keil et al., 1996; Marsden et al., 1984; Su et al., 1987). The secreted form as well as some membrane anchored form of gG are members of a novel family of viral chemokine binding proteins that bind a broad range of chemokines with high affinity (Bryant et al., 2003; Costes et al., 2005; Costes et al., 2006). In contrast, gG of HSV-1 lacks a secreted form. It exists as an unusually truncated protein compared to other gG proteins of alphaherpesviruses (McGeoch, 1990; McGeoch et al., 1987) and a chemokine binding activity is absent in HSV-1 gG. Deletion mutants of HSV-1 gG exhibited no attenuation of replication *in vitro* but had reduced virulence in experimental animals (Balan et al., 1994; Tran et al., 2000; Weber et al., 1987). The mechanisms involved in this attenuation are unclear.

HSV-1 gG was demonstrated to be implicated in virus entry through apical surfaces of polarized epithelial cells (Tran et al., 2000). However, the function of gG in HSV-1 infection of non-polarized cells remains to be elucidated. Recently, Nakamichi and colleagues (Nakamichi et al., 2001; Nakamichi et al., 2002; Nakamichi et al., 2000) described additional functions for BoHV-1 gG than the chemokine binding mechanism. They found early in infection less viable cells when cells were infected with a knockout of BoHV-1 gG mutant compared to parental BoHV-1 infected cells. Moreover, in the absence of BoHV-1 gG impaired cell junctions and reduced plaque sizes were observed. The authors suggest that BoHV-1 gG facilitates viral cell-to-cell transmission by stabilizing the cell structure and postponing apoptotic processes.

Much controversy has arisen about the egress pathway that has led to three theories. i) It is postulates that herpesvirus virions are fully assembled in the nucleus, acquiring their final envelope by budding at the inner nuclear membrane and escape from there via vesicle formation. These vesicles then pass the Golgi complex for final maturation of virions (Campadelli-Fiume et al., 1991). ii), The virion derived by budding at the inner nuclear membrane is speculated to be deenveloped by fusion of its envelope with the outer nuclear membrane releasing capsid and tegument into the cytoplasm. Capsids then bud into vesicles derived from the *trans*-Golgi network acquiring a final envelope and tegument (Skepper et al., 2001). iii) Both theories have recently been challenged by the discovery of dilated nuclear pores in infected cells (Wild et al., 2005). In the designated cytoplasmic envelopment route, capsids gain direct access from the nucleus to the cytoplasm via impaired nuclear pores. For acquiring an envelope and tegument, cytoplasmic capsids bud at membranes of the endoplasmic reticulum and the Golgi complex. In the nuclear pathway, which coexists in infected cells, virions originating by budding at the nuclear membrane are assumed to be intraluminally transported from the perinuclear space (PNS) into rough endoplasmic reticulum (RER) (Schwartz and Roizman, 1969) and further into the Golgi cisternae for packaging into transport vacuoles (Leuzinger et al., 2005; Wild et al., 2002).

The intraluminal pathway relies on a functional intracellular transport system that begins in the PNS involving RER and Golgi complex and ending at the cell periphery. Breakdown of this transport system may well lead to apoptosis. Since gG deletion mutants had been shown

to negatively affect cell viability, we hypothesized that gG may execute a protective role to prevent–or at least delay–breakdown of the transportation system.

In this study, we thus analyzed a gG-negative recombinant HSV-1 virus *in vitro* and observed unique characteristics of the gG-negative HSV-1 mutant that have not been previously reported. HSV-1 gG is required for efficient cell-to-cell spread in Vero cells whereas the growth kinetics is similar to wild-type HSV-1. Interestingly, in the absence of gG the Golgi complex was disassembled early, and virions accumulated within the PNS and RER. Therefore, gG is considered likely to be involved in sustaining of the Golgi complex to provide membranes for HSV-1 envelopment, and for facilitating viral transportation.

Material and Methods

Cells and viruses. HeLa cells stably expressing β 1,4-galactosyltransferase 1-green fluorescent protein (GT-GFP) were constructed as follows. The chimeric construct between GT and GFP was created using standard PCR protocols with the overlap extension technique (Ho et al., 1989). In one PCR the amino terminal part of GT including the cytoplasmic tail, the transmembrane domain and the stem region (up to amino acid 81) was amplified introducing a 5' EcoRI restriction site as well as a 3' overlap with GFP. The GFP sequence was amplified by PCR incorporating a 5' overlap with the GT and a 3' MluI restriction site. In a second PCR the two fragments were combined, digested with EcoRI and MluI and ligated into the plasmid pSFFVneo (Rohrer et al., 1995). The resulting plasmid was linearized with XbaI and stably transfected into HeLa cells using Lipofectin (Gibco, Bethesda, MD, USA) according to the manufacture's directions. Transfected cells were subjected to selection with neomycin (G418) at a concentration of 1 mg/ml and individual colonies were selected. The cells were maintained under selective pressure and the correct localization of the GT-GFP construct was verified by co-localization with endogenous GT. HeLa GT-GFP cells and african green monkey kidney (Vero) cells were maintained in Dulbecco's modified Eagle's medium (DMEM: Gibco) supplemented with 10% fetal calf serum (Gibco) at 37°C and 5% CO_2. Wild-type HSV-1 strain F and recombinant HSV-1 were propagated and titered on Vero cells.

Generation of recombinant viruses. Recombinant bacterial artificial chromosome (BAC) clones were generated using the galK positive/negative selection system as described

previously by Warming et al. (Warming et al., 2005). The pYEbac102ΔUS4, in which the entire US4 open reading frame in BAC HSV-1 (F) [pYEbac102 (Tanaka et al., 2003)] was replaced with a galK expression cassette, was constructed as follows. A linear DNA fragment was generated by PCR amplification of the galK gene including sequences complementary to those flanking US4 (primer forward, 5'-cgg gtt ggc aca aaa aga ccc cga tcc gcg tct gtg gtg ttt ttg gca tcC CTG TTG ACA ATT AAT CAT CGG CA-3'; primer reverse, 5'-att gtt ctt gct ttc cgc atg tgg gct ctc ccc atc ccc cgc ccc ata ccT CAG CAC TGT CCT GCT CCT T-3'; galK sequences are shown in capital letters). After DpnI digestion of the PCR template and gel purification, the DNA fragment was electroporated into competent *E. coli* strain SW102 maintaining pYEbac102. Electroporated bacteria were incubated at 32°C for 1 h and plated onto agar plates containing galactose to select for bacteria carrying the galK gene inserted into the US4 locus.

In order to generate the revertant virus pYEbac102 US4-HA, the galK negative selection was used. First, US4 sequence was amplified by PCR from pYEbac102 using a reverse primer containing nucleotides of HA tag (primer forward, 5'-gat ctc tag aAT CAT GTC GCA GGG CGC CA-3'; primer reverse, 5'-gat caa gct tag cat aat ctg gaa cat cat atg gat aCC CGC GTT CGG ACG GCA–3'; US4 sequences are shown in capital letters). Using this PCR product as a template, another PCR amplification was performed for the purpose of amplifying HA-tagged US4 with additional nucleotides homologous to the sequences flanking US4 (primer forward, 5'–CGG GTT GGC ACA AAA AGA CCC CGA TCC GCG TCT GTG GTG TTT TTG GCA TCA TGT CGC AGG GCG CCA–3'; primer reverse, 5'–ATT GTT CTT GCT TTC CGC ATG TGG GCT CTC CCC ATC CCC CGC CCC ATA CCC TAa gca taa tct gga aca tca tat gga–3'; US4 sequences are shown in capital letters). The linear PCR fragments were then electroporated into *E. coli* SW102 carrying pYEbac102ΔUS4. Transformants were incubated and streaked onto agar plates containing chloramphenicol and deoxy-galactose selecting for deoxy-galactose resistant colonies, which substituted the galK cassette by HA-tagged US4 gene.

To generate viruses CSΔUS4 and rCSUS4, purified recombinant DNA of pYEbac102ΔUS4 and pYEbac102 US4-HA, respectively, were cotransfected with pCre (plasmid harbouring Cre recombinase under CMV promoter) into Vero cells. Recombinant viruses were plaque purified three times, DNA of the resulted viruses were isolated and verified by PCR amplification, restriction enzyme fragment analysis and Southern blotting with a digoxigenin-labeled probe. The probe was either derived from a US4 PCR product or from a galK PCR product.

Virus purification. Cell culture flasks with a 150 cm^2 growth area of confluent Vero cells were infected at MOI of 1. The infected cells were scraped off culture dishes between 36 and 48 h after infection, and lysed by sonication. Cellular debris was removed by centrifugation and virus was purified by ultracentrifucation through a 25% sucrose cushion for 3 h. The pellet containing virus particles was resuspended in Hank's Balanced Salt Solution (HBSS). The quality of the virion preparation was verified by electron microscopy.

Antibodies. The anti-HA monoclonal antibody was obtained from Santa Cruz Biotechnology. The polyclonal antiserum raised against the HSV-1 tegument protein US3 and the monoclonal antibody against HSV-1 gG were gifts from Dr. Bernhard Roizman, University of Chicago, USA. MAb 3114 specific for HSV-1 gE was kindly provided by Nigel Stow, Institute of Virology, Glasgow, United Kingdom. Anti-ICP4 was purchased from Advanced Biotechnologies Inc, Columbia, Maryland, USA. Peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibodies were from Sigma, Buchs, Switzerland. Goat anti-mouse antibodies conjugated to Alexa-594 was obtained from Invitrogen, Basel, Switzerland.

Western blot analysis of virions and cell lysates. Infected or noninfected cells were scraped into lysis buffer (100 mM NaCl, 20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.5% NP-40 and protease inhibitor cocktail [Invitrogen, Basel, Switzerland]). Protein concentration of lysed cells or purified virions was measured using Bradford reagent (BioRad, Reinach, Switzerland). After addition of sample buffer (50 mM Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate [SDS], 1% β-mercaptoethanol, 20% glycerol, and 1% bromphenolblue), samples were boiled for 10 min. Ten micrograms of purified viral proteins or cell lysate were separated by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes. Blots were blocked with 5% low-fat milk in phosphate-buffered saline (PBS) and probed with primary antibodies as indicated in *Results*. Subsequently, blots were incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody and visualized on X-ray film using chemiluminescence. For reprobing of membranes, blots were incubated in stripping buffer (62.5 mM Tris-HCl [pH 6.8], 100 mM β-mercaptoethanol, and 2% SDS) for 30 min at 70°C, subsequently blocked and reprobed.

Plaque assays of cell-to-cell spread. For plaque morphology Vero cells were infected with an MOI of 0.001. After 2 h, the virus was removed and cells were overlayed with medium containing 1% caboxymethylcellulose. At day 1, 2, and 3 post infection (pi), cells were fixed in PBS containing 3% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min, blocked with 2% bovine serum albumin (BSA) in PBS for 30 min and then stained with mouse anti-gE serum MAb 3114 and goat anti-mouse immunoglobulin G (IgG) antibodies conjugated with horseradish peroxidase. Plaques were visualized after the addition of peroxidase substrate diaminobenzidine hydrochloride. The area of each plaque was determined by capturing images using a CCD camera attached to a Zeiss Axiovert S 100 microscope and quantifying the size of 25 plaques per sample using National Institutes of Health Imaging software.

One-step growth analysis. Vero cells were infected with CSΔUS4, rCSUS4 or HSV-1 at an MOI of 0.1. At various times after infection, cells and supernatants were harvested. Cells were lysed by three rounds of freeze-and-thaw. Cell debris was then removed by centrifugation. Virus titers were determined by endpoint titration of virus stocks on Vero cells.

Low-temperature transmission electron microscopy. Vero cells were infected with the indicated viruses (MOI of 5) and prepared for electron microscopic investigations as previously described in detail (Wild et al., 2002). Briefly, cells were frozen in a high-pressure freezing unit (HPM; BAL-TEC Inc., Balzers, Liechtenstein) and then substituted with acetone. Subsequently, samples were fixed with 0.25% glutaraldehyde and 0.5% osmium tetroxide at temperatures between -30°C and +2°C to achieve a good contrast of membranes (Wild et al., 2001), and embedded in Epon at +4°C followed by polymerization at +60°C for 2.5 days. Ultrathin sections were analyzed in a CM12 Philips transmission electron microscope equipped with a slow-scan charge coupled device camera (Gatan, Pleasanton, CA).

Morphometric analysis. Volume and surface area of the Golgi complex were estimated by morphometric analysis on randomly selected cells at a final magnification of 89'500 applying the point counting method (Weibel, 1979). The data were calculated on the basis of the equations Vvg=Pg/Pcy, and Svg=Ig/Pcy*d, whereby Vvg is the volume density, Svg the surface density of the Golgi complex, Ig are intersections on Golgi membranes, Pg are points

hitting the lumen of Golgi complex, Pcy are points hitting the cytoplasm, and d is the test line length. The data are expressed as μm^3 volume or as μm^2 surface area per 1000 μm^3 cytoplasm.

Confocal microscopy. For indirect immunofluorescence studies, HeLa GT-GFP cells grown on glass cover slips. At the stated times cells were fixed in PBS containing 3% paraformaldehyde for 15 min at room temperature and then with cold acetone for 2 min at -20°C. Residual aldehyde groups were quenched with 50 mM glycine. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 5 min and incubated with PBS containing 2% bovine serum albumin (BSA). Cells reacted for 60 min at room temperature with monoclonal antibody anti-ICP4 HSV-1 diluted in PBS/BSA, afterwards incubated for 60 min with goat anti-mouse antibodies conjugated to Alexa-594. Cell nuclei were counterstained with 1 µg/ml DAPI (Roche, Reinach, Switzerland) for 5 min. Samples were embedded in fluorescence mounting medium (DakoCytomation, Glostrup, Denmark) and analyzed by using a confocal laser scanning microscope (SP1, Leica, Mannheim, Germany). Images were deconvolved employing a blind deconvolution algorithm using the program suite Huygens Essential (SVI, Hilversum, The Netherlands).

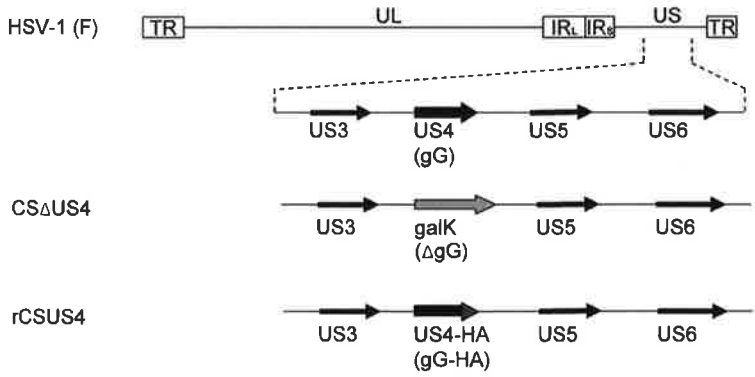

Fig. 1. **Schematic representations of the DNA sequence arrangements in the genomes of wild-type HSV-1 and mutant strains CSΔUS4 and rCSUS4.** The top line represents the arrangement of the HSV-1 (F) genome with the unique long (UL) and unique short (US) regions flanked by the terminal repeat (TR) and internal repeat (IR) regions. Depicted below is the region of HSV-1 DNA containing open reading frames US3, US4 (gG), US5 and US6. CSΔUS4 was generated by replacing the open reading frame of US4 with a galK cassette. rCSUS4 was derived from pYEbac102ΔUS4 by substituting the galK cassette by a cloned fragment harbouring gG with an HA tag.

Results

Construction of US4 deletion and repair virus mutants. To address the *in vitro* function of gG in HSV-1, a gG deletion mutant (CSΔUS4) was generated. As detailed in *Materials and Methods* and illustrated in Fig. 1, we replaced the entire coding sequence of US4 with a galK expression cassette. PCR was used to amplify the galK cassette encompassed by 50-bp regions homologous to sequences flanking HSV-1 US4. The PCR product was introduced into the HSV-1 BAC plasmid pYEbac102 by homologous recombination in *E. coli* (Warming et al., 2005). The US4 open reading frame was effectively removed from its start codon to its stop codon. The resulting BAC was named pYEbac102ΔUS4, which contains the galK cassette instead of the US4 coding sequence. The revertant mutant pYEbac102 US4-HA was restored by inserting the HSV-1 US4 gene tagged at its carboxyterminus with a hemagglutinin (HA) epitope into pYEbac102ΔUS4 by homologous recombination. The PCR product of US4-HA tagged sequence and homologous flanking sequences was electroporated into competent *E. coli* harbouring pYEbac102ΔUS4. Homologous recombination resulted in restoring the entire sequence of US4 with an additional HA tag to its original location in the virus BAC. To recover the recombinant viruses CSΔUS4 and rCSUS4, pYEbac102ΔUS4 and pYEbac102 US4-HA, respectively, were cotransfected along with Cre recombinase expression plasmid into Vero cells. CSΔUS4 grew without the use of complementing cells confirming earlier data that US4 is not an essential gene for virus replication in cell culture. Viruses were plaque purified and the viral genomes were analysed. Accurate insertion of the recombinant fragments within the HSV-1 genome was confirmed by PCR and Southern blot (Supplementary Figure 1) before the viruses were functionally assessed in the course of the present study.

Characterization of the US4 deletion mutant. To confirm the deletion and repair of the US4 open reading frame, the incorporation of gG into virus particles was examined by immunoblotting (Fig. 2). Using virus particles purified from infected Vero cells, we could confirm that gG was clearly present in wild-type and rCSUS4 virus particles, whereas gG was not detectable in CSΔUS4 virions (Fig. 2A). As expected, immunoblotting with an anti-HA antibody gave only a positive signal with rCSUS4 (Fig. 2B). In contrast, the expression of gE protein did not show any significant differences in the purified virus particles (Fig. 2C). Furthermore, the detection of gG expression in infected Vero cells was also performed by immunofluorescence (Supplementary Figures 2 and 3) confirming the results obtained by

western blotting. The deletion of gG gene was designed in such a manner that the expression of the neighbouring genes should not be affected. Transcription of both the upstream US3 gene and US4 gene terminate at the same polyadenylation site located downstream of the US4 open reading frame. Demmin et al. (Demmin et al., 2001) reported that the expression of the upstream gene US3 was altered in PRV after insertion into the US4 gene. Thus, the observed phenotype in PRV was due to the reduced US3 expression and not due to the loss of gG. To ensure that the deletion of US4 in CSΔUS4 did not affect expression of the US3 gene product, lysates of infected Vero cells were examined (Fig. 3). Vero cells infected with HSV-1, CSΔUS4, and rCSUS4 were harvested at 16 h and 24 h pi. Immunoblots were probed with an anti-US3 polyclonal antibody and proved the expression of US3 in approximately equivalent amounts in the examined infected cells. As a loading control, the same immunoblots were reprobed with an anti-ICP4. US4 deletion in CSΔUS4 had no apparent effect on the accumulation of US3 protein and, consequently, all observed phenotypes were considered to be due to the deletion of US4.

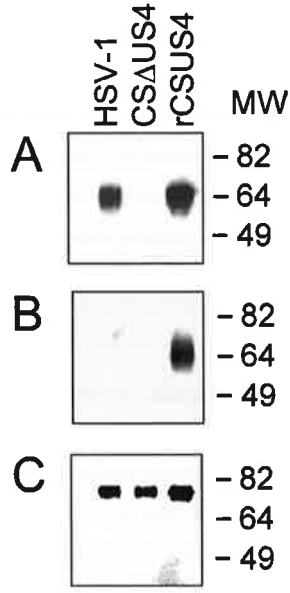

Fig. 2. **Western blot analysis of purified wild-type and recombinant virus particles.** Vero cells were infected either with HSV-1, CSΔUS4, or rCSUS4. After 48 h, cells were disrupted and virus particles were purified through sucrose cushions. Membranes were probed for gG, gG-HA and gE with antibodies directed against gG (A), HA-tag (B), and gE (C).

Growth properties of the US4 deletion mutant. Previous work demonstrated that gG in BoHV-1 is required for efficient cell-to-cell spread in Madin-Darby bovine kidney cells (Nakamichi et al., 2000). Thus, we evaluated the rate of replication of CSΔUS4 by plaque assay in order to determine whether or not US4 is involved in viral direct cell-to-cell spread of HSV-1. Vero cells were infected with HSV-1, CSΔUS4 or rCSUS4 and covered with a semisolid overlay to prevent readsorption of released virus to neighbouring uninfected cells. As shown in Fig. 4, cell-to-cell transmission was considerably reduced in CSΔUS4 infected Vero cells compared to HSV-1 infected cells. Quantitative analysis of viral plaques showed that the plaque area of CSΔUS4 infected cells was nearly half of the area of those formed in HSV-1 infected cells or in rCSUS4 infected cells at 24 h pi. At 48 h and 72 h pi the produced plaques of CSΔUS4 have, on average, a 30% reduced size compared to wild-type and revertant virus (Fig. 4B). No plaque size difference was observed in the revertant mutant rCSUS4 versus HSV-1 indicating that the cell-to-cell spreading defect observed in CSΔUS4 is indeed attributable to the lack of US4. These findings indicate that gG may be necessary for effective cell-to-cell spread. However, the smaller plaque areas of virus could also be caused by reduced ability to replicate. Therefore, the growth kinetics of HSV-1, CSΔUS4 and rCSUS4 were compared. Infected Vero cells were lysed at different time points and virus was quantified by endpoint titration. Fig. 5 illustrates the results of the virus titers obtained at various times pi. On average, CSΔUS4 reached a 10% and 7.5% lower virus yield compared to HSV-1 at 24 h and 36 h pi, respectively. At early times of infection, virus yield did not differ between HSV-1 and CSΔUS4. Growth kinetics of revertant mutant rCSUS4 was similar to wild-type. Taken together, although CSΔUS4 produced significantly smaller plaques than wild-type virus, only a small reduction in replication was observed compared to HSV-1. Consequently, gG is required for an efficient cell-to-cell spread but not for an high titre replication in cell culture.

Accumulation of CSΔUS4 particles in the PNS and endoplasmic reticulum. To address the implications of US4 on the observed viral phenotype, infected cells were examined in more details by electron microscopy. At various times, intracellular virus particles were enumerated in 20 randomly selected cells to detect possible defects in the egress pathway in the absence of gG (Table 1). Cells infected with CSΔUS4 showed normal morphogenesis of nucleocapsids and similar numbers of budding capsids at the inner nuclear membrane compared to HSV-1 infected cells. However, there were dramatic differences in the number of

enveloped virions in the PNS and RER of infected cells (Fig. 6). While in HSV-1 infected cells 5% to 9% of total counted virus particles were located in the PNS, this fraction was significantly increased statistically from 18% to as much as 44% in CSΔUS4 infected cells at the given times (Table 1). Furthermore, CSΔUS4 was not only retained in the PNS, it also accumulated within the RER. In wild-type HSV-1 infected cells approximately 6% to 11% of total counted virus particles were encountered in the RER, whereas in the absence of gG roughly threefold more particles were detected in the RER. Cells infected with rCSUS4 showed all stages of virus maturation and no accumulation of virus particles in the PNS or RER were observed. No obvious differences could be seen between wild-type and rCSUS4 infected cells.

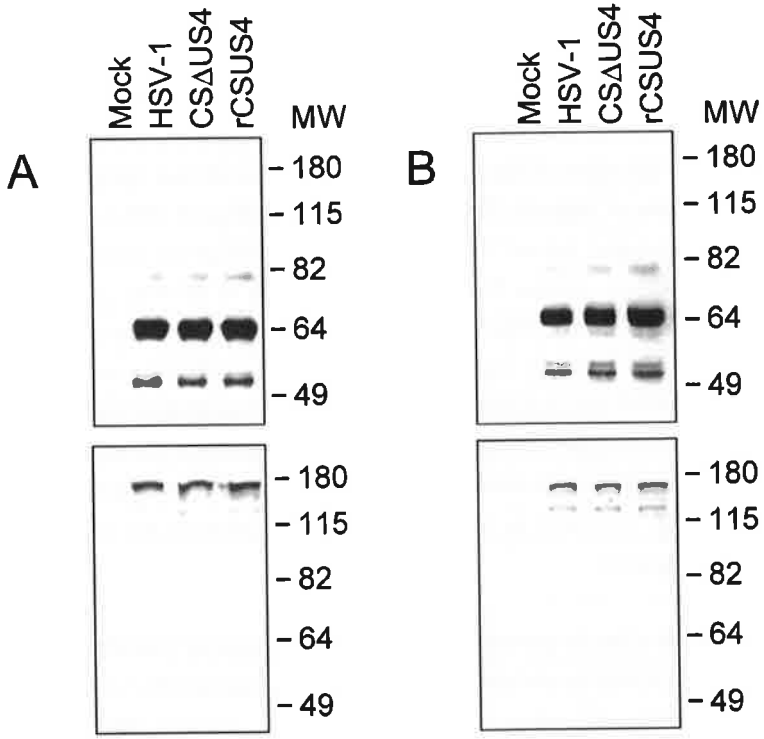

Fig. 3. **US3 protein expression levels of wild-type and recombinant HSV were analyzed by Western blot using an anti-US3 antibody.** Infected Vero cells were lysed 16 h pi (A) or 24 h pi (B). Vero cells were either mock infected, or infected with HSV-1, CSΔUS4, or rCSUS4. The membranes in (A) and (B) were first probed with anti-US3 antibodies (upper panel) and after stripping reprobed for ICP4 protein with anti-ICP4 antibodies (lower panel) as a loading control.

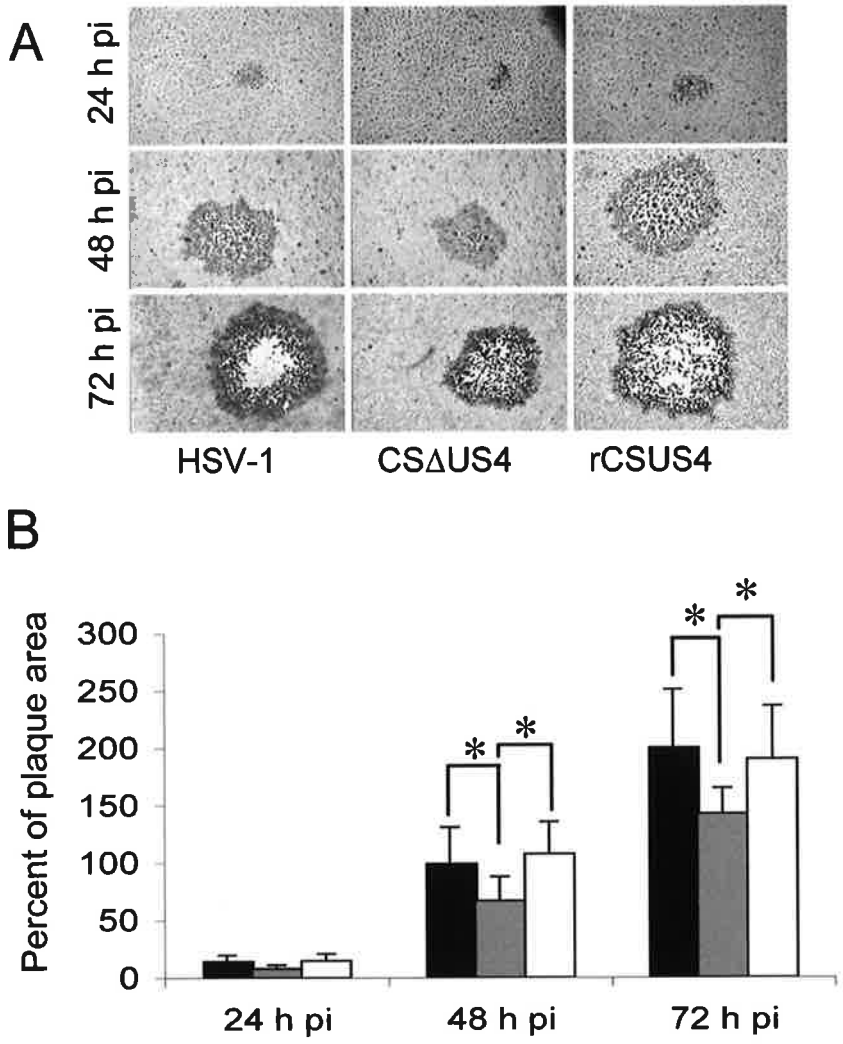

Fig. 4. **Plaque morphologies of wild-type HSV-1 and recombinants.** (A) Vero cells were infected with HSV-1, CSΔUS4, or rCSUS4 using an MOI of 0.001. After 2 h, virus containing medium was removed and cells were overlaid with semisolid medium. At the indicated times, cells were fixed and stained with anti-HSV-1 gE antibodies and peroxidase-conjugated secondary antibodies. (B) Twenty plaques produced by each virus were photographed and subjected to image analysis to quantify the area of the plaques. The average area of plaques formed by HSV-1 48 h pi was defined as 100%. Mean and standard deviations are shown. HSV-1 (black bars), CSΔUS4 (grey bars), and rCSUS4 (open bars). *$p<0.001$.

Large numbers of naked capsids accumulated in the cytoplasm of CSΔUS4 infected Vero cells. However, less than 10% of CSΔUS4 particles were observed in Golgi complex and vesicles, whereas enveloped virions within Golgi cisternae and vesicles were numerous (up to 54%) in HSV-1 infected Vero cells. Since CSΔUS4 capsids were often found to be associated with Golgi membranes, the small number of virus particles within vesicles in CSΔUS4 infected cells seems not due to an inhibited transport of capsids within the cytoplasm. It seems that budding at Golgi membrane *per se* is impaired. Of particular interest, the majority of Golgi complexes in the examined ultrathin sections were markedly reduced in CSΔUS4 infected cells compared to wild-type infected cells.

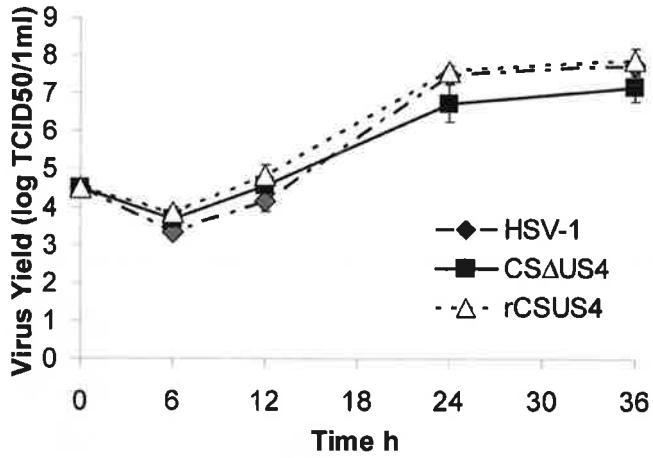

Fig. 5. **One-step growth kinetics of wild-type and HSV-1 recombinants.** Vero cells were infected with HSV-1, CSΔUS4, or rCSUS4 at an MOI of 1. At the times indicated, the titers of progeny viruses were determined as TCID50/ml. Mean and standard deviation of the results from three independent experiments are shown.

Surface area and volume of the Golgi complex are reduced in CSΔUS4 infected cells. Electron microscopy suggested that the Golgi complex of infected cells is rudimentary in the absence of gG. In HSV-1 infected cells several cisternae with lateral dilations were found while only few small cisternae were present in CSΔUS4 infected cells at 16 h pi (Fig. 7). To confirm these observations, the surface area and volume of the Golgi complex were

determined by morphometric analysis (Weibel, 1979). 30 cells infected either with mock, wild-type, or CSΔUS4 were chosen at random. Early in infection, no significant differences of the Golgi surface area and volume could be detected. However, at 16 and 20 h pi, cells infected with CSΔUS4 showed a Golgi surface area that was only 40% of the Golgi surface area of wild-type infected cells (Fig. 8A). The volume of the Golgi was even more drastically reduced by approximately 65% to 74% in the absence of gG compared to HSV-1 at 16 h and 20 h pi, respectively (Fig. 8B). Since different sections through the Golgi complex were taken, the measured data strongly varied among cells and as a consequence, standard deviations are relatively high. Nevertheless, the surface area as well as the volume of the Golgi complex was significantly smaller in CSΔUS4 infected cells compared to cells infected with wild-type virus ($p<0.001$).

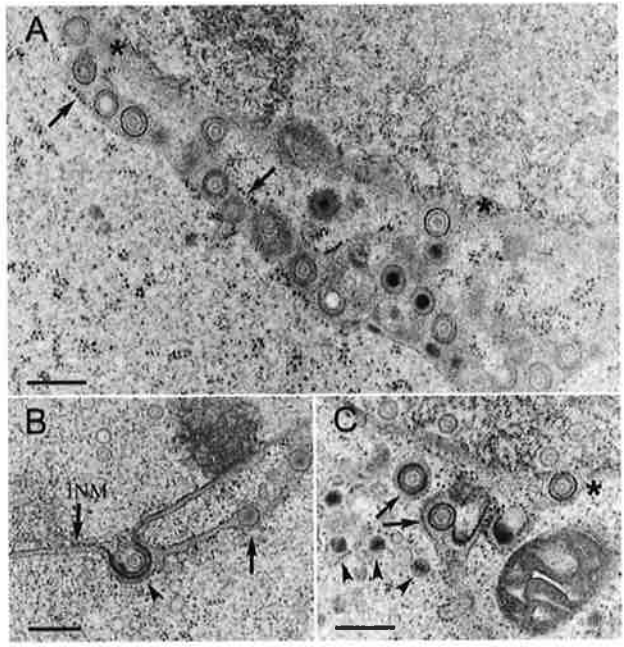

Fig. 6. **Section through Vero cells infected with CSΔUS4 (A), HSV-1 (B), and rCSUS4 (C) with an MOI of 5 16 h pi.** (A) Accumulation of enveloped virions within RER cisterna (arrows) adjacent to the PNS (tangentially sectioned, asterisks), a typical feature in the absence of gG. (B, C) Budding of capsids (arrowhead) at the inner nuclear membrane (INM) was rarely observed. The number of enveloped virions within the RER (arrows) is low in HSV-1 (B) and rCSUS4 (C) infected cells, whereas the number of naked capsids (arrowheads) in the cytoplasm was equal to that in CSΔUS4 infected cells. Bars, 500 nm.

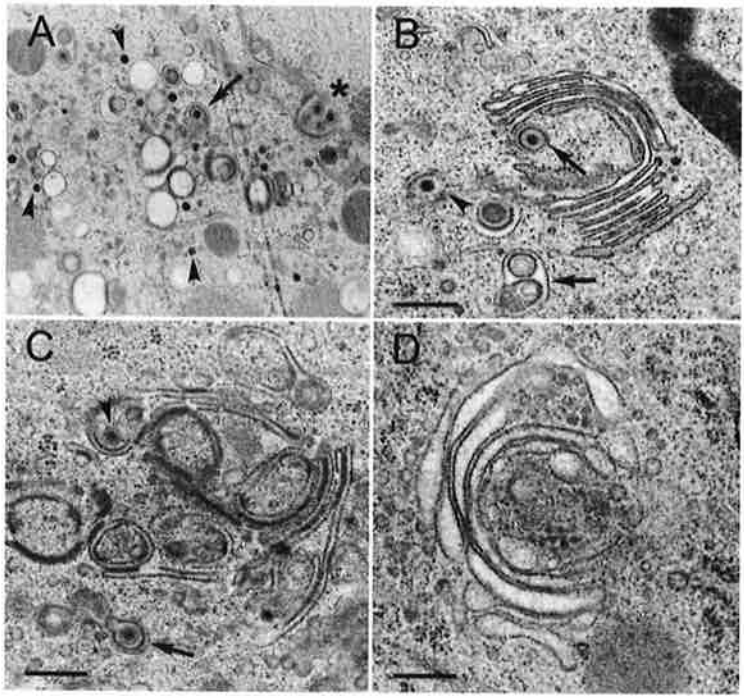

Fig. 7. **Golgi fields of Vero cells 16 h after infection.** (A) In CSΔUS4 infected cells the Golgi complex is disassembled rarely containing virions (arrow) but naked capsids (arrowheads) are distributed throughout the cytoplasm, and virions got stuck in the PNS (asterisk). (B, C) In HSV-1 (B), and rCSUS4 (C) infected cells the Golgi complex comprises several cisternae associated with enveloped virions (arrow) and budding capsids (arrowheads). (D) The Golgi complex in mock infected cells consisting of several cisternae. Bars, 500 nm.

Structural changes of the Golgi complex after CSΔUS4 infection. To re-evaluate the morphometric data of the Golgi complex by a different method, confocal microscopy was performed. For this purpose, HeLa GT-GFP cells stably expressing a fusion of galactosyltransferase membrane domain and green fluorescence protein (GT-GFP) were used to visualize the Golgi complex. The fluorescent Golgi probe GT-GFP has been characterised before in detail and used to study Golgi membrane dynamics (Cole et al., 1996). Infected or mock infected HeLa GT-GFP cells were fixed after different time points and stained with antibodies against ICP4 to ascertain whether or not infected cells were being analyzed. In mock infected cells typical tubular structures of the Golgi complex were observed at all analysed time points (Fig. 9A). Infection with HSV-1 did not affect the Golgi structure

significantly in the course of infection up to 20 h pi. However, after incubation for 24 h, GT-GFP fluorescence signal had almost completely disappeared. Only a few small GT-GFP dots remained in the cytoplasm of HSV-1 infected cells (Fig. 9B). Similar results were obtained by scanning rCSUS4 infected cells showing Golgi complex in a typical ribbon-like shape localized in a perinuclear region up to 20 h of infection (Fig. 9D). Fluorescence signal of GT-GFP was also vanished 24 h pi in rCSUS4 infected cells as in wild-type HSV-1 infected cells. By contrast, in the absence of gG, the Golgi complex was disorganized and appeared as numerous punctuated structures scattered throughout the cytoplasm as early as 10 h after exposure to CSΔUS4 virus (Fig. 9C). Further incubation of CSΔUS4 infected cells did not change the dotted pattern of Golgi cisternae. The GT-GFP fluorescence signal remained equal early and late in infection in CSΔUS4 infected cells in contrast to HSV-1 infected cells. Taken together, our results indicate that gG has a protective effect for a lasting integrity of the Golgi complex during HSV-1 infection.

Table 1. Distribution of virus particles in sections of Vero cells infected with wild-type HSV-1 or CSΔUS4.

Subcellular Locl	Mean number of particles/ cells (%)[a]							
	12 h pi		16 h pl		20 h pi		24 h pi	
	HSV-1	CSΔUS4	HSV-1	CSΔUS4	HSV-1	CSΔUS4	HSV-1	CSΔUS4
INM[b]	1.7(10.5)	0.6(2.9)	0.5(2.8)	0.8(2.9)	0.4(1.5)	0.7(1.9)	0.2(0.9)	1.2(3.5)
PNS	1.5(8.9)	8.9*(43.6)	1.0(6.3)	4.7*(17.9)	1.5(5.5)	7.2*(21.0)	1.2(5.3)	8.6*(25.1)
RER	1.9(11.7)	5.6*(27.2)	1.2(7.3)	7.5*(28.6)	1.7(6.5)	10.1*(29.5)	1.8(8.0)	9.8*(28.6)
Golgl/ Vesicle	6.4(39.1)	0.6*(2.7)	6.9(43.5)	2.0*(7.6)	14.1(53.5)	2.2*(6.3)	5.5(24.3)	2.3(6.8)
Cytoplasm[c]	4.9(29.8)	4.8(23.5)	6.4(40.1)	11.3(42.9)	8.7(33.0)	14.2(41.3)	13.9(61.5)	12.3(36.0)

[a]Virus particles present in infected Vero cells were counted in electron micrographs of 20 randomly sampled cells. The numbers represent the mean number of virus particles per compartment, and the numbers in parentheses indicate the percentage of virus particles per compartment.
[b]capsids budding at the inner nuclear membrane (INM).
[c]unenveloped capsids in the cytoplasm.
*Differences in mean numbers of CSΔUS4 compared to HSV-1 at a given time were significant ($p<0.001$) as assessed by pair wise testing applying Student's *t* test.

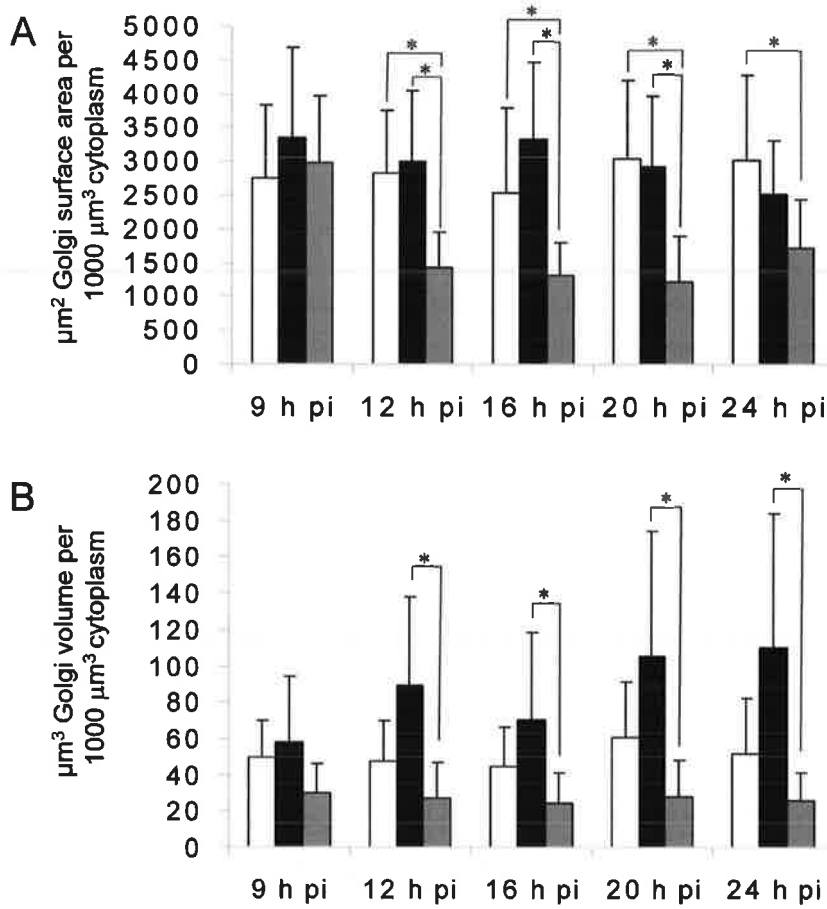

Fig. 8. **Quantitative analysis of surface area (A) and volume (B) of Golgi complex during virus multiplication.** Vero cells were infected with HSV-1, CSΔUS4, or mock at an MOI of 5. At the times indicated, the cells were frozen and prepared for electron microscopy. 30 randomly selected cells were photographed and analyzed using the point counting method. Surface area of the Golgi complex was significantly smaller (*$p<0.001$) in CSΔUS4 (grey bars) infected cells compared to HSV-1 (black bars) infected cells or mock (open bars) infected cells after 12 h pi. Also the volume of the Golgi complex was diminished in CSΔUS4 infected cells during virus replication. Mean and standard deviation of the results are shown.

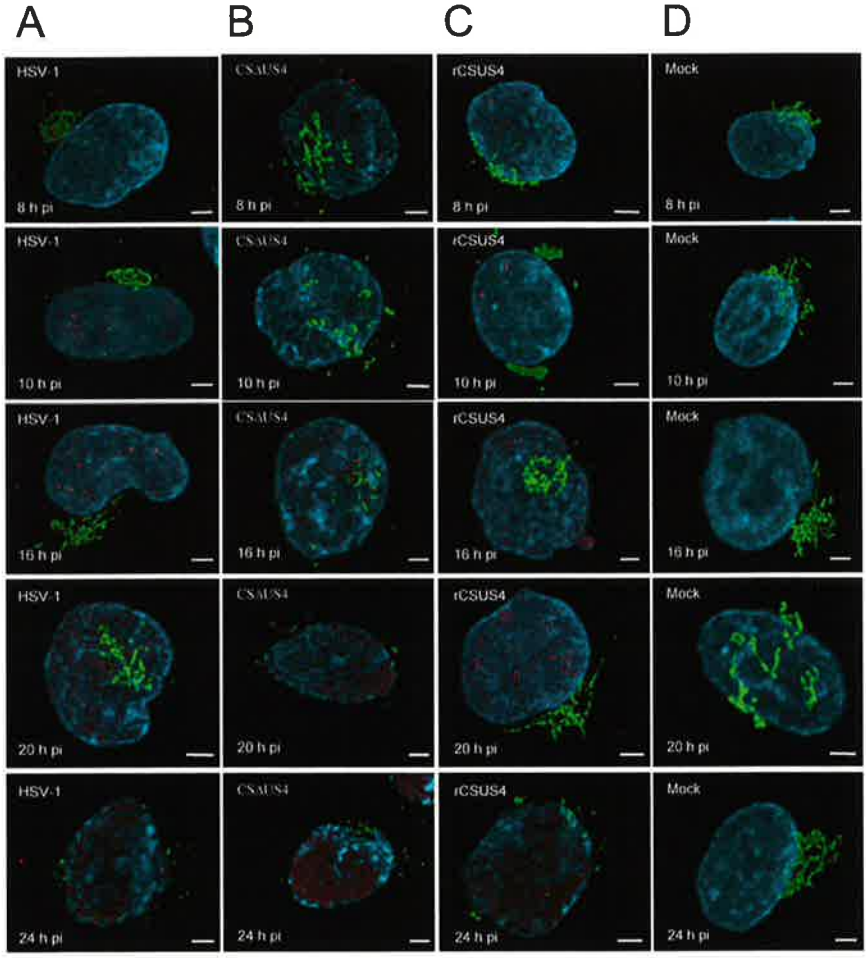

Fig. 9. **Three-dimensional distribution of Golgi apparatus (green; GT-GFP) obtained by confocal microscopy of HeLa GT-GFP cells.** Cells infected with HSV-1 (A), CSΔUS4 (B), rCSUS4 (C), or mock infected (D) were fixed and stained 8, 10, 16, 20 or 24 h pi. Infection of cells was ascertained by immunolabeling of ICP4, an early viral protein (red). Nuclei were stained with DAPI (blue). Bars, 3 μm.

Discussion

In herpesvirus infected cells the Golgi complex is required to modify the carbohydrate structure of glycoproteins, to provide membranes for capsid envelopment, and for formation of vacuoles that transport virions towards the cell periphery. Herpesvirus egress can thus be considered as a variation of a common secretory pathway (Palade, 1975). It has been reported that infection with wild-type HSV-1 induces fragmentation and dispersal of the Golgi apparatus late in infection. This phenomenon, observed in Vero, HEp-2, and BHK cells, coincided with the maximum rate of viral reproduction (Avitabile et al., 1995; Campadelli et al., 1993). Since Golgi membranes are needed for viral envelopment *per se* and for the formation of transport vacuoles, fragmentation of the Golgi complex late in infection is a possible consequence of exhaustion of Golgi membranes. Interestingly, in the present study infection with HSV-1 gG deficient mutant led to premature diminution and disassembly of Golgi structures beginning as early as 10 h after inoculation. In contrast, in cells infected with wild-type virus the Golgi complex remained intact until 21 h pi. This phenomenon was observed in Vero as well as in HeLa GT-GFP cells (Figs. 8 and 9). Changes in phenotypes in gG deletion mutants may be due to reduced expression of the upstream US3 gene rather than to the absence gG (Demmin et al., 2001). The US3 message polyadenylation signal is located downstream of the gG gene (Rixon and McGeoch, 1985). Accordingly, insertions or deletions in the gG genes of these viruses might also affect the expression of US3. In our study, the coding sequence of HSV-1 gG was replaced by a galK cassette (Fig. 1) and Western blot experiments confirmed that the accumulation of US3 was not impaired in the constructed CSΔUS4 mutant (Fig. 3).

Less than 10% of total virions were detected in Golgi cisternae or Golgi-derived vesicles in CSΔUS4 infected cells, whereas in HSV-1 infected cells up to 53.5% of total virus particles were counted in Golgi cisternae or Golgi-derived vesicles (Table 1). However, the total virus yield of CSΔUS4 was equal to that of wild-type HSV-1 (Fig. 5). Consequently, the majority of virions contributing to the yield of infectious virus have originated by budding at the inner nuclear membrane. Thus, virions may acquire all essential components including tegument proteins and glycoproteins by budding at the inner nuclear membrane. Indeed, many glycoproteins have been demonstrated to be present at nuclear membranes (Avitabile et al., 1994; Baines et al., 2007; Browne et al., 1996; Hutchinson et al., 1995; Oravcova et al., 2000; Rychlowski, 2000). Virions within the PNS were also shown to contain glycoproteins (Baines

et al., 2007; Torrisi et al., 1992) and the essential tegument protein VP16 (Naldinho-Souto et al., 2006).

In addition to the disassembly of the Golgi complex, we found virions accumulating within the PNS and RER (Fig. 7) in the absence of gG. Most of these virions have probably derived by budding at the inner nuclear membrane. Accumulation of virions in the PNS and RER due to an increased budding activity at the nuclear membrane is unlikely since the number of budding events counted in CSΔUS4 infected cells is similar to that of wild-type infected cells. In the deenvelopment-reenvelopment hypothesis, the primary envelope of virions is believed to fuse with the outer nuclear membrane releasing capsids and tegument into the cytoplasm (Smith, 1980; Stackpole, 1969). In this context, accumulation of virions in the PNS and RER would be the result of suppressed deenvelopment that would result in diminished numbers of capsids in the cytoplasm. In fact, CSΔUS4 and wild-type infected cells exhibited similar numbers of naked capsids in the cytoplasm (Table 1). The significant accumulation of CSΔUS4 virions in the PNS and RER cannot be explained by the de-envelopment hypothesis. It is, however, consistent with the vesicle formation theory and the recently proposed dual-pathway hypothesis. However, if virions exit the PNS via vesicles formed at the outer nuclear membrane no naked capsids will be expected in the cytoplasm. The dual pathway theory suggests that alphaherpesvirus particles follow two alternative routes for egress, which coexist in the same infected cell (Leuzinger et al., 2005). In the cytoplasmic envelopment route, capsids are suggested to exit the nucleus via impaired nuclear pores and gain direct access to the cytoplasm. Dilated nuclear pores were reported both in HSV-1 and BoHV-1 infected cells (Leuzinger et al., 2005; Wild et al., 2005). After transport of cytoplasmic capsids to the Golgi apparatus, capsids are wrapped by Golgi membranes or bud into Golgi derived vacuoles. In the nuclear envelopment pathway, fully enveloped particles are formed by capsids budding at the nuclear membrane.

The presence of virions within the RER cisternae and Golgi cisternae strongly suggests that virus particles are intraluminally transported from the PNS to the Golgi complex via RER (Leuzinger et al., 2005). Connectivity between PNS, RER and Golgi cisternae was shown in BoHV-1 infected cells (Wild et al., 2002). It is likely that disassembly of the Golgi complex will block transport of virions from the PNS into Golgi cisternae causing accumulation of virions in PNS and RER as observed in CSΔUS4 infected cells. This conclusion is supported by the fact that brefeldin A treatment, which causes a breakdown of the Golgi apparatus, also

resulted in accumulation of virions in PNS and RER of infected cells (Dasgupta and Wilson, 2001). It is thus considered likely that gG has a protective role for maintaining the Golgi complex, and consequently, the functionality of the intracellular transport system.

Virions transported into Golgi cisternae are packaged into transport vacuoles by fission from Golgi membranes (Wild et al., 2002) which need to be replaced. Furthermore, cytoplasmic capsids budding at Golgi derived vacuoles or Golgi cisternae (Homman-Loudiyi et al., 2003) require also large amounts of membranes upon progress of viral multiplication. In wild-type HSV-1 infected cells, it is likely that membranes of the Golgi complex were eventually used up for envelopment of cytoplasmic capsids at a late stage of infection, since fragmentation of Golgi complex is regularly seen late (24 h pi) in wild-type infected cells (Fig. 9). Consequently, less virus particles were found in Golgi-derived vesicles, whereas the number of cytoplasmic capsids was increased in HSV-1 infected cells after 24 h of infection. CSΔUS4 infected cells featured a significant smaller surface area and volume of the Golgi complex as well as a significant reduction of enveloped virus particles within Golgi cisternae compared to wild-type infected cells suggesting that successful budding events were strongly impaired by degraded Golgi structures.

Formation of transport vacuoles by the Golgi complex is also required for efficient viral transport to the plasma membrane or cell junctions allowing cell-to-cell spread (Johnson and Huber, 2002; Johnson et al., 2001). Because the Golgi complex was disassembled in CSΔUS4 infected cells relatively early in infection, and hence was unable to sort virions into transport vacuoles, it was not surprising that CSΔUS4 showed a significantly reduced efficiency in cell-to-cell spread compared to HSV-1 (Fig. 4). The small plaque sizes in CSΔUS4 infected monolayers are assumed to be a consequence of premature Golgi breakdown and reduced cell-to-cell spread. Similar observations have been reported from cells infected with gG deficient mutants of BoHV-1, equine herpes virus 4 and PRV (Demmin et al., 2001; Huang et al., 2005; Nakamichi et al., 2000).

In conclusion, infection of cells with the HSV-1 gG deletion mutant, CSΔUS4, resulted in disassembly of the Golgi complex early in infection. Accumulation of enveloped virions within the PNS and RER indicates suppressed intraluminal transportation into Golgi cisternae due to breakdown of the Golgi complex. Disassembly of the Golgi complex led also to diminished formation of vacuoles containing virus destined for transportation to the cell

periphery that is assumed to be related to inefficient cell-to-cell spread. However, yields of infected virus particles were not significantly affected implying that virions derived by budding at the inner nuclear membrane are infective. Therefore, gG is suggested to play an essential role in protecting the Golgi complex whose membranes are used for both envelopment and formation of transport vacuoles. Furthermore, gG may be used to study aspects of the secretory pathway system.

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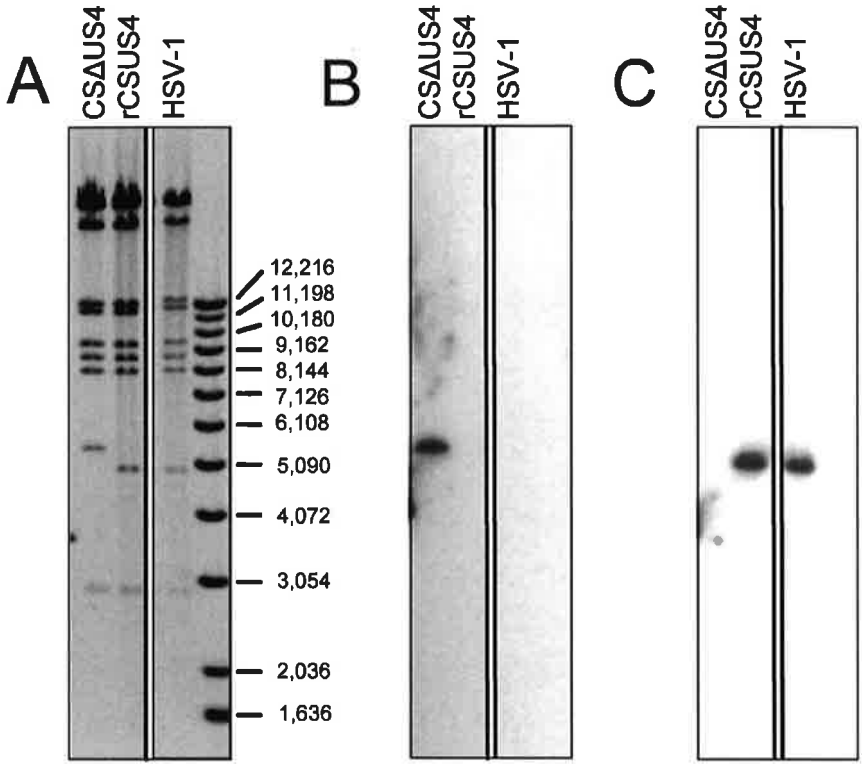
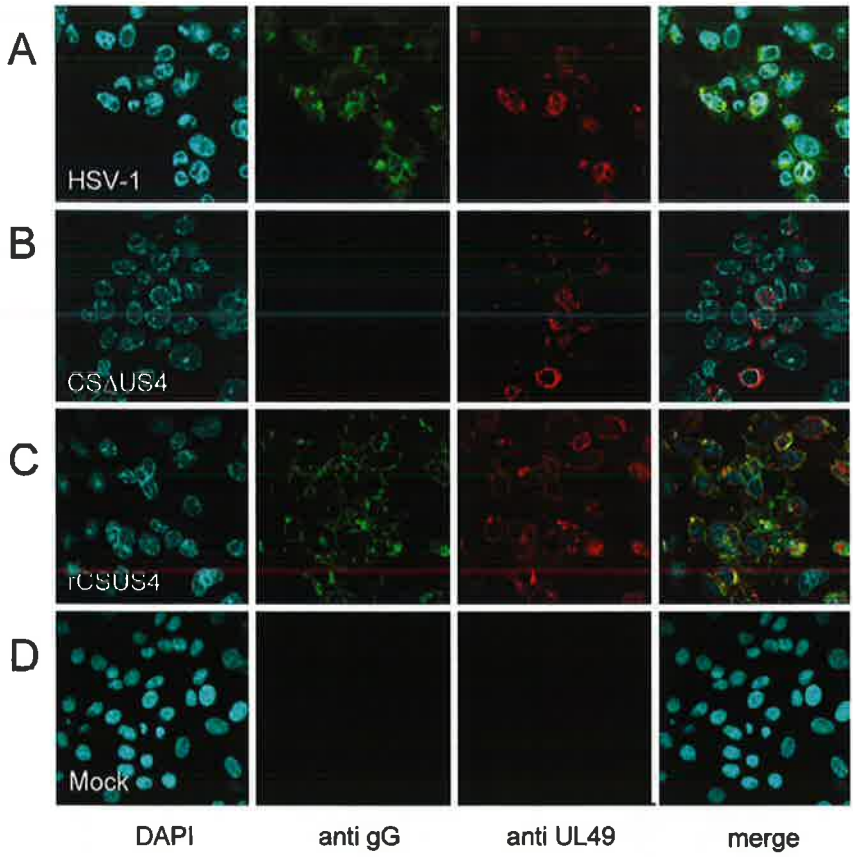
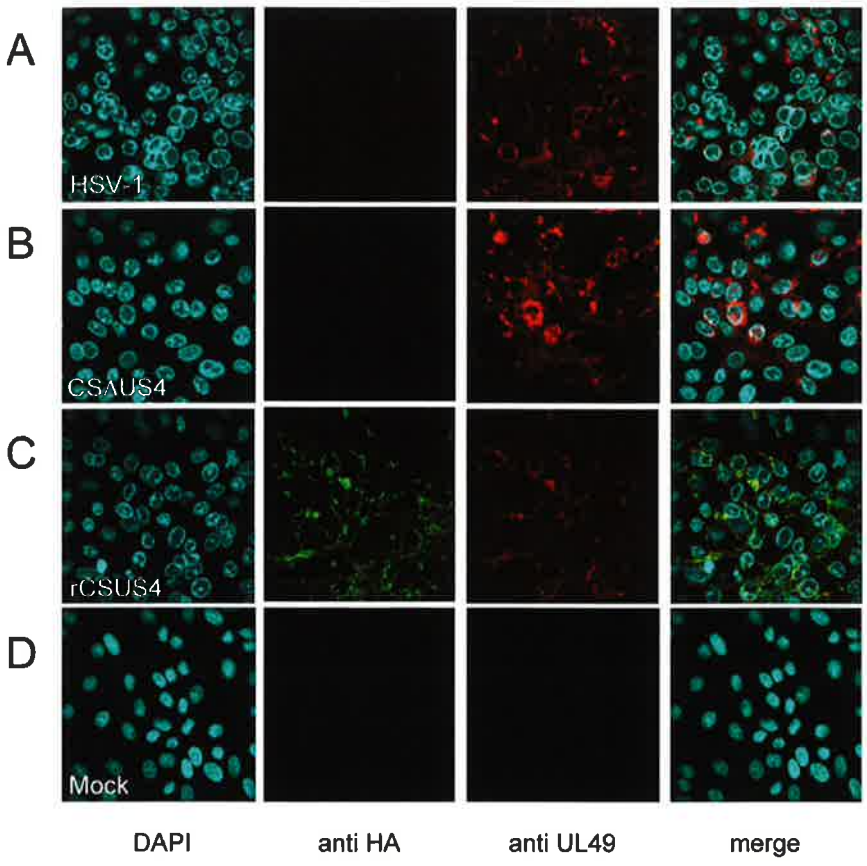
Supplementary Figures



Supplementary Figure 1. **Genomic analysis of wild-type HSV-1 and mutants.** (A) Wild-type HSV-1 and recombinant virus were used to infect Vero cells at an MOI of 1. Virus particles were harvested 48 h pi and viral genome was isolated. One microgram aliquots of viral DNA were digested with restriction endonuclease HindIII. The digested DNA was resolved on a 0.7% agarose gel. Due to the insertion of the galK expression cassette in CSΔUS4 the 4.9 kb fragment in the digested wild-type HSV-1 and rCSUS4 genome was upshifted to 5.4 kb in the digested CSΔUS4 genome. (B, C) For Southern blot analyses, the DNA was transferred onto nylon membrane and hybridized with a DIG-labeled galK probe (B) or with a DIG-labeled gG probe (C). The galK probe hybridized to the 5.4 kb DNA fragment in CSΔUS4, which is absent in rCSUS4 and HSV-1 DNA. The gG probe identified a 4.9 kb DNA fragment in rCSUS4 and HSV-1 DNA.



Supplementary Figure 2. **Immunofluorescence staining of infected Vero cells.** Cells were infected with wild-type HSV-1 (A), CSΔUS4 (B), rCSUS4 (C) or mock (D). Infected Vero cells were fixed at 24 h pi, and stained with monoclonal anti-HSV-1 gG antibodies and polyclonal rabbit antibodies against HSV-1 UL49. Cell nuclei were counterstained with DAPI.



Supplementary Figure 3. **Immunofluorescence staining of Vero cells infected with wild-type HSV-1 (A), CSΔUS4 (B), rCSUS4 (C) or mock (D).** Infected Vero cells were fixed at 24 h pi, and stained with monoclonal anti-HA antibodies and polyclonal rabbit antibodies against HSV-1 UL49. Cell nuclei were counterstained with DAPI.

Chapter 5

Conclusions and Perspectives

Our proposed dual pathway model

The mechanism by which herpesviruses leave the host cell is controversially discussed for many years. Two general pathways have been hypothesized for virion egress: the vesicle formation model and the reenvelopment model. The controversy centres on how virus particles leave the perinuclear space to the exterior of the infected cells (see General Introduction). Both models of egress find support from individual studies and neither model has been decisively disproved. The weakness of both proposed models is their inability to demonstrate how virions leave the perinuclear space. Fusion events between the viral envelope and the outer nuclear membrane as well as formation of vesicles at the outer nuclear membrane could never been convincingly shown. Moreover, mutant viruses deleted in different membrane proteins all produce cytoplasmic capsids. Therefore, the unanswered question is how cytoplasmic capsids originate. We tried to answer this question by studying egress of BoHV-1, HSV-1 and mutant viruses of HSV-1 deleted either in gK or gG. We have chosen gK, because it is the only glycoprotein essential for egress, yet it was unclear for which step gK was required. The function of gG in HSV-1 was unknown but gG had been demonstrated to affect cell-to-cell spread in other alphaherpesviruses.

We have challenged the current models of herpesvirus envelopment by finding that the outer nuclear membrane neither forms vesicles enclosing virus particles nor fuses with the viral envelope. Instead, we found two new coexisting routes how herpesviruses leave the nucleus. Our model, named the dual pathway model, proposes on the one hand that capsids in the nucleus can gain directly access to the cytoplasm through dilated nuclear pores and on the other hand, that capsids budding at the inner nuclear membrane are intraluminally transported from the perinuclear space to the Golgi complex via endoplasmic reticulum (Fig. 1).

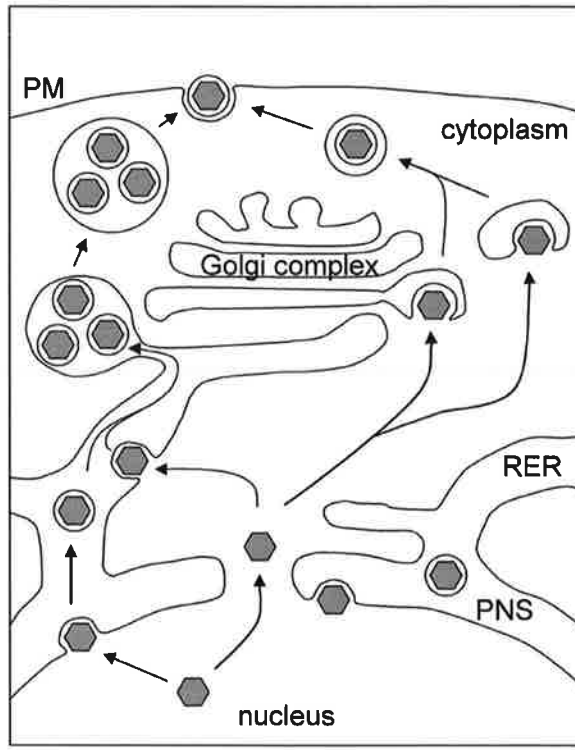


Fig. 1. **Schematic drawing of the dual pathway model of herpesvirus envelopment.** In the cytoplasmic transport pathway, capsids leave the nucleus via impaired nuclear pores and approach cellular membranes from the cytoplasmic side, inducing budding. In the luminal transport pathway, capsids bud through the inner nuclear membrane and are transported from the perinuclear space (PNS) via rough endoplasmic reticulum (RER) cisternae into Golgi cisternae for packaging into transport vacuoles containing one or more virions. Virus particles were released from the cell by exocytosis. PM, plasma membrane.

Loss of integrity of the nuclear envelopment was found in cells infected with BoHV-1 as well as with HSV-1 indicating a validity of this attribute during infection for a broad range of herpesvirus. We found gaps in the nuclear membrane of infected cells measured up to 1,900 nm at 14 h post infection. They were bordered by the inner nuclear membrane continuing into the outer nuclear membrane and thus were assumed to represent impaired nuclear pores lacking nuclear pore complex structures. Interestingly, nuclear material together with capsids protruded through dilated nuclear pores into the cytoplasm suggesting that capsids gain directly access to the cytoplasm through these gaps in the nuclear envelope. The existence of

our proposed egress route through dilated nuclear pores was further confirmed in cells infected with a gK deletion mutant of HSV-1 (CSΔgK). Strikingly, no enveloped virus particles were found in the perinuclear space in the absence of gK, yet the cytoplasm contained large numbers of naked capsids. Consequently, CSΔgK infected cells demonstrated that cytoplasmic capsids occurred without primary budding events at the inner nuclear membrane. Examination of the nuclear surface of CSΔgK infected cells revealed huge gaps in the nuclear envelope, which were similar in wild-type infected cells. Nuclear matrix protruded into the cytoplasm through these gaps reinforcing the idea that the naked capsids found in the cytoplasm were stemming from capsids transported from the nucleus through these enlarged nuclear pores. The reenvelopment model as well as the vesicle formation model consider that budding at the inner nuclear membrane is the prerequisite for the release of cytoplasmic capsids. Therefore, this finding further contradicts the validity of the reenvelopment model and of the vesicle formation model.

Cytoplasmic capsids would acquire their envelope by budding at any cellular membrane before leaving the cells by exocytosis. Therefore, interactions of capsids with the outer nuclear membrane represent budding events from the cytoplasmic side rather than fusion events. In the absence of gK, capsids were found to be unable to complete budding processes at any cellular membrane, whereas transportation of capsids towards cellular membranes was not suppressed (Fig. 2). Therefore, the present hypothesis is that the function of gK is to complete envelopment of capsids at the inner nuclear membrane as well as at the endoplasmic reticulum membrane and Golgi membranes.

The second coexisting route of our proposed dual pathway model involves envelopment at the inner nuclear membrane followed by intraluminal transport through the endoplasmic reticulum and Golgi apparatus and finally leaving the cell using the secretory machinery. Virus particles within the perinuclear space, endoplasmic reticulum and Golgi cisternae support our idea of intraluminal transport. Moreover, the phenotype of cells infected with a gG deletion mutant of HSV-1 (CSΔUS4) is fully consistent with the intraluminal transport concept.

CSΔUS4 infected cells display an outstanding phenotype with four distinguished attributes (Fig. 3): (1) accumulation of virions in the perinuclear space and rough endoplasmic reticulum; (2) only few virions within Golgi cisternae or within vacuoles derived from Golgi complex; (3) reduced plaque formation and (4) Golgi complex was disassembled early in infection (Fig. 3). If we reevaluate these observations in the light of the dual pathway model,

the diminution in intraluminal transport of virions could be linked directly to the accumulation of nucleocapsids in the perinuclear space and rough endoplasmic reticulum, which in turn was the result of the disassembly of the Golgi complex. Consistently, HSV-1 infected cells treated with brefeldin A, which causes breakdown of Golgi complex, also resulted in accumulation of virions in the perinuclear space and rough endoplasmic reticulum (Dasgupta and Wilson, 2001). In contrast, in the reenvelopment model accumulation of virus particles would result from abolished fusion of viral membrane with the outer nuclear membrane. However, the finding of similar numbers of naked capsids in the cytoplasm of CSΔUS4 and wild-type HSV-1 infected cells refute this interpretation. Reduced plaque formation seen in the absence of gG was directly linked to inefficient cell-to-cell spread, which in turn was due to diminished formation of vacuoles bearing virions for transportation to the cell periphery. Diminished envelopment of cytoplasmic capsids by Golgi membranes was again owing to the breakdown of the Golgi complex in the absence of gG.

Further, the intraluminal transportation of virus particles predicts that formation of intact virions is essentially completed by envelopment at the inner nuclear membrane, whereas the reenvelopment model argues that perinuclear virions were not fully tegumented. The infectivity of the gG deletion mutant of HSV-1 demonstrates that capsids budding at the inner nuclear membrane acquire all essential components including tegument proteins and glycoproteins. In the absence of gG, a significant lower number of virions were found in Golgi complex and vesicles derived thereof compared to wild-type. However, the virus titers from wild-type and gG deletion mutant were similar.

Obviously, our results indicate that the Golgi complex plays an important role in the maturation of herpesviruses. Virus infection represents cellular stress involving enormous DNA and protein synthesis. It has been shown that Golgi membranes were full of newly synthesised viral membrane proteins (Farnsworth and Johnson, 2006; Wisner and Johnson, 2004). Therefore, it is possible that herpesviruses encode protein(s) which help the cellular membranes to sustain such a huge influx of membrane proteins. gG is a potential candidate for facilitating stability of Golgi membranes, since in the absence of gG, the Golgi complex was disassembled early in infection.

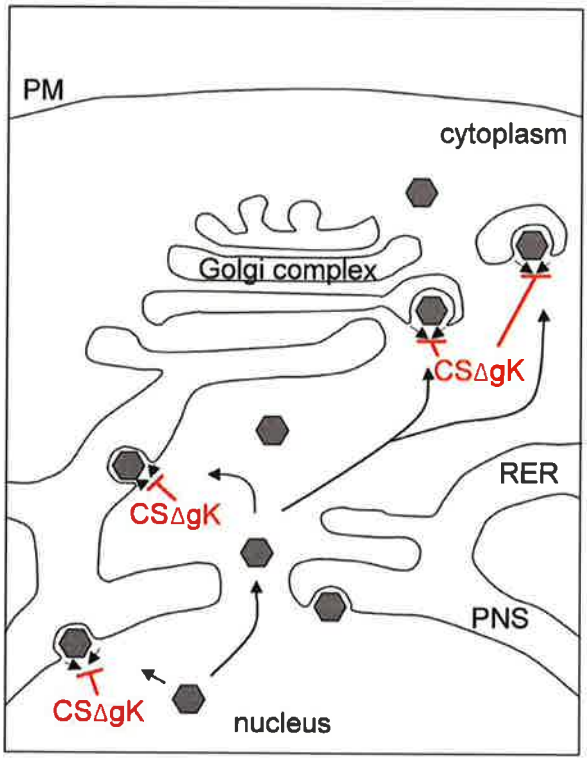


Fig. 2. **Schematic drawing of the intracellular phenotypes of CSΔgK infected cells.** Envelopment of nucleocapsids and of cytoplasmic capsids is defective in the absence of gK. PM, plasma membrane; RER, rough endoplasmic reticulum; PNS, perinuclear space.

Our dual pathway model, overcomes the major inconsistencies of the two other concepts; vesicle formation or fusion at the outer nuclear membrane. For example the phospholipid composition of membranes of released HSV-1 virion was compared to those of the host cell membranes (van Genderen et al., 1994). Based on relative levels of phosphatidylserine and sphingomyelin, the envelope of extracellular virions was found to mimic neither those of Golgi-derived membranes nor those of nuclear membranes. The divergence in lipid composition is best explained by the dual site of envelope origin. Also the presence of *cis*- and *medium*-Golgi markers (Miranda-Saksena et al., 2002) as well as cytoplasmic host

proteins on extracellular virions (Dienes et al., 1987; Varnum et al., 2004) can be explained with our proposed dual pathway theory.

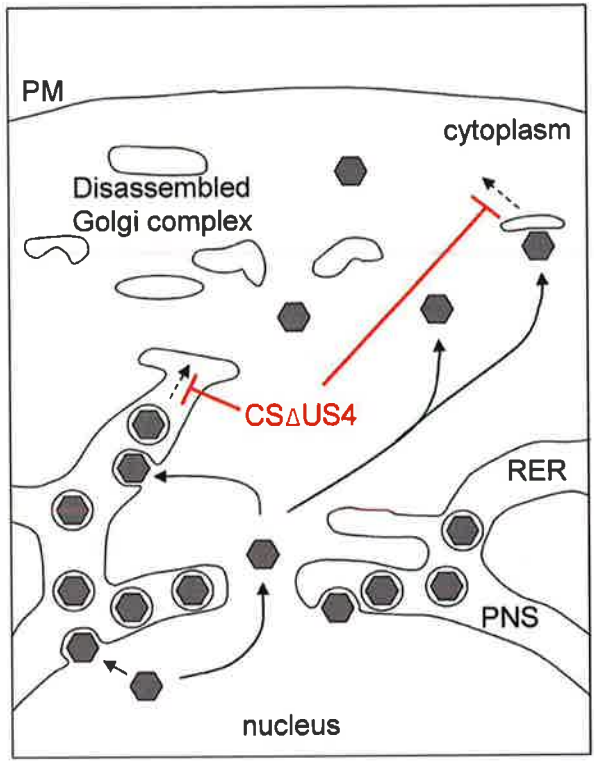


Fig. 3. **Schematic drawing of the intracellular phenotypes of CSΔUS4 infected cells.** Wrapping of virus particles with Golgi membranes is reduced due to breakdown of Golgi complex, which is induced in the absent of gG early in infection. PM, Plasma membrane; RER, rough endoplasmic reticulum; PNS, perinuclear space.

Perspectives

The results of infection with CSΔUS4 disclosed the necessity to sustain the Golgi complex during infection. So far, gG is the only herpesvirus protein known to serve such a function. Therefore, gG may be also used to study other aspects of the secretory pathway. The Golgi

complex undergoes rapid fragmentation and reassembly during mitosis (Puri et al., 2004). If our proposed function of gG as a stabilising agent of Golgi complex is correct, mitosis may be reduced or blocked in cells expressing gG. Furthermore, it would also be interesting to investigate the integrity of Golgi complex in cells expressing gG in the presence of compounds, such as brefeldin A, which disassemble the Golgi complex.

If the dual pathway model is correct, cytoplasmic capsids will be absent when the nuclear pores are intact. Moreover, intact nuclear pores will only permit intraluminal transport of virions from the perinuclear space to the extracellular space. Therefore, the key evidence required to support the dual pathway model is the identification of proteins that are required for nuclear pore dilation. The finding of proteins that abolish transportation of capsids either to budding sites of the inner nuclear membrane or through the nuclear pore can also help resolve this issue. Furthermore, identification of essential tegument proteins and glycoproteins on virions within the perinuclear space is a key to discerning the route of viral egress.

An interesting approach would be to target the budding process to special cellular compartments, such as endoplasmic reticulum or Golgi membranes. For this, recombinant viruses would be generated which encode gK with an endoplasmic reticulum retention motif. Provided that gK is retained in the endoplasmic reticulum and not present in the Golgi or plasma membrane, envelopment of capsids should only occur at endoplasmic reticulum membranes. Moreover, if recombinant viruses containing gK with an endoplasmic reticulum retention motif could be propagated and would use exclusively the luminal transportation, it would strongly support the dual pathway model.

Ultimately, confirmation of the dual pathway theory would come from analysis of the lipid composition of the viral envelope. For this purpose the extracellular virions must be separated into two populations, one population are virions derived from the intraluminal transport, the second population are virions utilizing the impaired nuclear pores and were wrapped by cytoplasmic membranes. If the dual pathway is correct, the first population will feature a lipid composition like the nuclear membrane, whereas the second population will possess a lipid composition similar to that of Golgi membranes. Unfortunately, this issue is difficult to resolve since the two populations of virions cannot yet be purified to homogeneity.

It is of interest to investigate the functions of additional viral proteins, which could be important for egress. Furthermore, it has to be elucidated how capsids traverse the cytoplasmic matrix to gain access to desired intracellular domains for further viral maturations. Overall, this would help to disclose the complete details of herpesvirus egress.

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List of Publications

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